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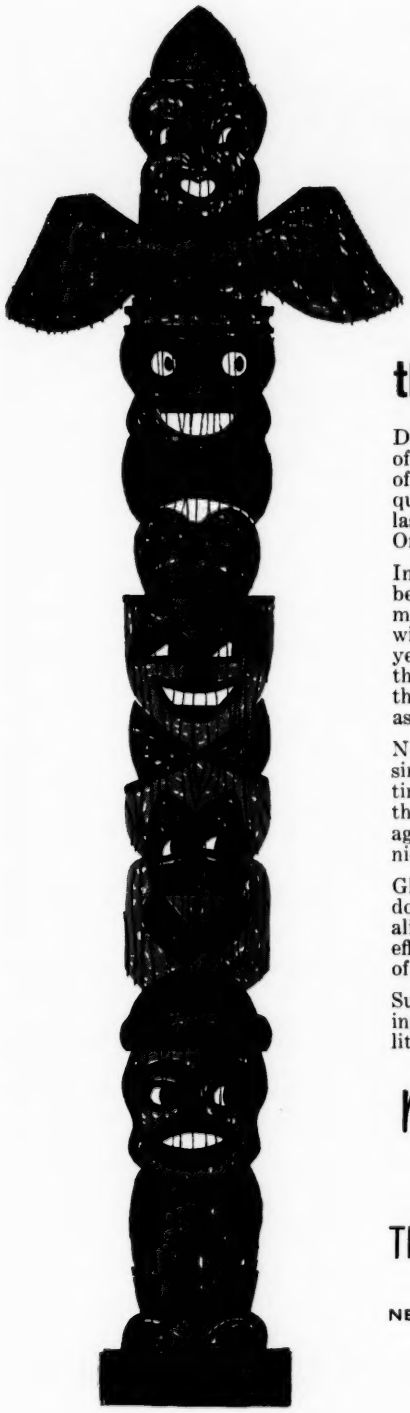

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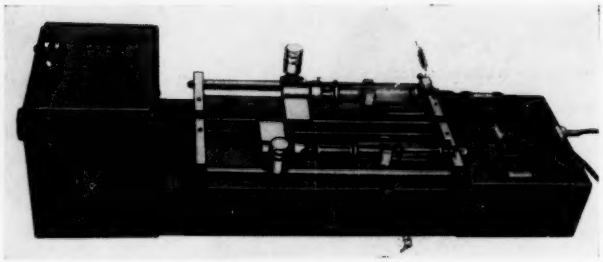

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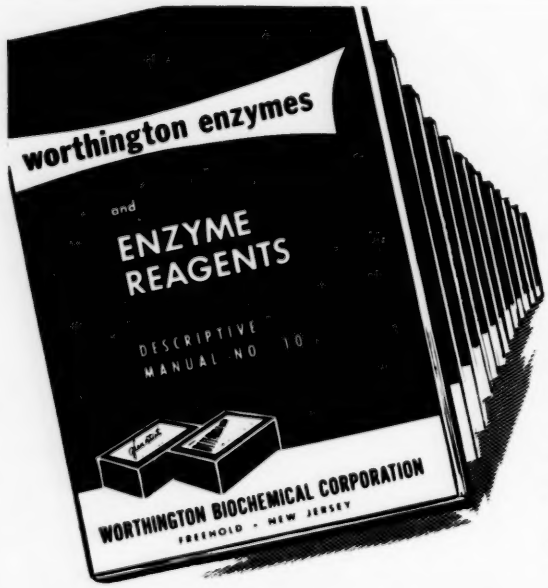

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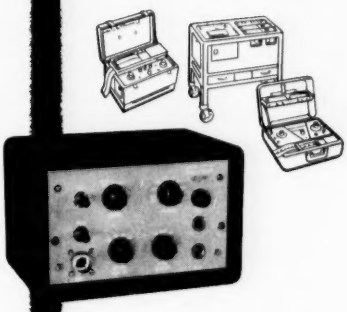

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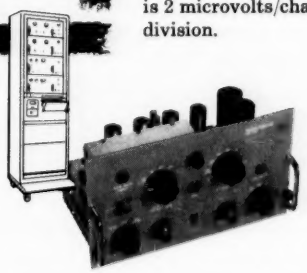

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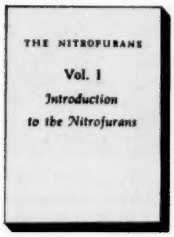

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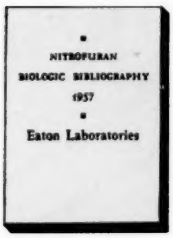

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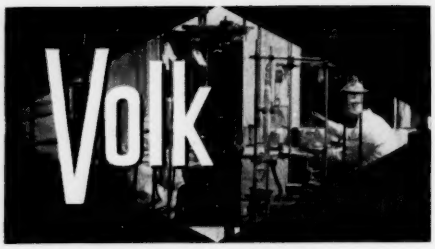

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THE ACID MUCOPOLYSACCHARIDE AND 5-HYDROXYTRYPTAMINE CONTENT OF HUMAN THROMBOCYTES IN RHEUMATOID ARTHRITIC AND NONARTHRITIC INDIVIDUALS *

By GRACE P. KERBY AND S. M. TAYLOR

(From the Department of Medicine, Duke University School of Medicine, Durham, N. C.)

(Submitted for publication January 27, 1959; accepted February 19, 1959)

The occurrence of acid mucopolysaccharides in human thrombocytes has been reported from this laboratory (1). The present continuing study had as its purpose further qualitative characterization and a study of the amounts of the acid mucopolysaccharides present in thrombocytes of patients with rheumatoid arthritis as compared to nonarthritic individuals. Because of the acidic nature of the mucopolysaccharides, it was proposed further to measure the amount of 5-hydroxytryptamine present in the same thrombocyte preparations, to determine if any correlation might exist in the content of these two classes of substances.

The overall results of the qualitative studies suggested the probability that a mixture of acid mucopolysaccharides is present in human thrombocytes. The amount present in thrombocytes per unit of blood was significantly greater in patients with nonrheumatoid inflammatory processes than in the control group, but the amount present per unit of thrombocytes was essentially the same in all categories of patients studied. There was no correlation between amount of acid mucopolysaccharides and amount of 5-hydroxytryptamine in human thrombocytes in the present study.

EXPERIMENTS

Blood was obtained by venipuncture from hospitalized patients,[1] using siliconed syringes and dispensing rapidly in approximately 9 ml. amounts to siliconed tubes containing 1 ml. of 1 per cent disodium ethylenedinitrilo tetraacetate (Eastman) in 0.7 per cent aqueous sodium chloride solution. The blood level in each tube was marked before centrifuging, after which thrombocytes were separated from the blood by the method of Dillard, Brecher and Cronkite (3). The volume of blood used was measured accurately by replacement to mark on the finally emptied tubes, correcting for known volume of anticoagulant; the blood sample usually totaled 50 to 60 ml. from each patient.

The thrombocyte buttons were drained after second centrifuging and the inner walls of each tube were wiped dry with gauze. Saline wash was omitted to avoid trauma to and loss of thrombocytes in a third centrifuging. The thrombocytes were resuspended smoothly [2] in 25 ml. 0.14 M NaCl in 0.02 M NaOH. Partial disruption of thrombocytes started spontaneously on brief standing at room temperature and was completed by sonication in a Raytheon Sonic Oscillator at 9 KC. per second for 10 minutes. Duplicate 9 ml. aliquots of the clear sonicate were then carried through perchloric acid precipitation and the subsequent steps for recovery of acid mucopolysaccharides by the method of Bollet, Seraydarian and Simpson (4), the final hexuronic acid content of the fraction being determined by the carbazole color reaction of Dische (5). A 0.5 ml. aliquot of the sonicate was used for biuret determination by the method of Jayle, Boussier and Badin (6).[3] Duplicate 3 ml. ali-

* This work was supported by grants from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, United States Public Health Service; and the American Heart Association.

[1] As in previous investigations (2), three groups of patients were studied. The groups were as previously defined and consisted essentially of Group A with no evidence of inflammatory disease, Group B with significant but nonrheumatoid inflammatory processes and Group C with unequivocal active rheumatoid arthritis.

[2] Preparations uniformly resuspended smoothly. Clumping occurred only with damaged thrombocytes and such preparations were discarded.

[3] Biuret determination (expressed as mg. of standard human serum albumin, A.R.C., Squibb) was used as a method for quantitation of thrombocytes in the present study to circumvent the inaccuracies inherent in methods for counting thrombocytes. Since the study was a comparative one, the use of a standard method for recovery of thrombocytes introduced a fairly uniform factor of loss in recovery into all groups. The amount of biuret positive material found in thrombocytes from 100 ml. of blood was of an order of magnitude comparable to that observed by the others [summarized by Zucker and Borreli (7)], on conversion of their data from protein nitrogen per unit of thrombocytes to protein content per usual number of thrombocytes in 100 ml. of normal blood. The likelihood of significant contamination of thrombocytes with traces of plasma was discounted since biuret determinations on 1:100 dilutions of plasma supernates revealed no significant differences in plasma biuret level between the three groups studied, in contrast to the sig-

quots of the sonicate were used for extraction of 5-hydroxytryptamine using the fluorometric method of Udenfriend, Weissbach and Clark (8), with final extraction into 0.1 N hydrochloric acid and measurement of 5-hydroxytryptamine by use of an Aminco Spectrophotofluorometer with activation at 295 mμ and fluorescence at 335 mμ. Standards of 1, 2 and 3 μg. of serotonin creatinine sulfate (kindly furnished by Abbott Laboratories), were run through the entire extraction procedure with every determination and recovery from thrombocytes was calculated from the standard curve obtained, converting the final values to 5-hydroxytryptamine.[4]

Thrombocytes used for additional biochemical studies were prepared as for the determination of hexuronic acid by the carbazole method. Using thrombocytes prepared from a patient with no evidence of inflammatory disease, hexuronic acid was determined in parallel by the carbazole method and by a modification of the orcinol method of Brown (9). In each of two other similar thrombocyte preparations, parallel determinations were made of the hexuronic acid content by the carbazole method and of the hexosamine content by a modification (10) of the method of Elson and Morgan.

To test the susceptibility of the thrombocyte fraction to hydrolysis by testicular hyaluronidase, thrombocytes from a patient with peptic ulcer were carried through the sonication step noted previously. One aliquot was then incubated for 255 minutes at 37° with 600 units of Alidase® (Schering Corp., commercial testicular hyaluronidase), as were control preparations of a thrombocyte aliquot in buffer and Alidase® in buffer. All preparations were then carried through perchloric acid precipitation, dialysis against running water and the subsequent steps of the Bollet procedure for recovery of acid mucopolysaccharides.

For paper chromatography of acid mucopolysaccharides, thrombocytes were carried through perchloric acid precipitation and water dialysis steps, then dried in a Virtis Freeze-Dryer and taken up in small amounts of water. Paper chromatography was carried out by methods previously described (11, 12). The authors are indebted to Dr. Karl Meyer for the standard preparations of heparitin sulfate and of chondroitin sulfate A, B and C used for comparison with test samples. Commercial heparin (Liquaemin®, Organon, Inc.) was used.

For paper chromatography of hexosamines, thrombocytes were carried through perchloric acid precipitation and water dialysis steps, then brought to pH 5.0 and approximately 0.04 buffer molarity by the addition of 0.1 M phosphate buffer adjusted to pH 5.0 with hydrochloric acid. Acid mucopolysaccharides were then precipitated by the addition of cetyl trimethyl ammonium bromide (13) and taken up in a small amount of 4 N hydrochloric acid. After eight hours of hydrolysis in sealed ampules in flowing steam, the sample was dried by gentle airjet in a jar containing solid sodium hydroxide, taken up in water and adjusted to pH 7.5 to 8.0 by addition of a trace of 0.1 N sodium hydroxide. Partial purification of hexosamines was then accomplished by passage over a Dowex 50 (H^+ form) column.[5] The final eluate was dried by airjet as before and taken up in a minute amount of water. The thrombocyte material and the chondroitin sulfate and hexosamine standard preparations were then hydrolyzed in sealed capillary tubes with equal volumes of 2 per cent ninhydrin in water containing 4 per cent pyridine at 100° C. for 30 minutes (14). The hydrolyzed materials were then chromatographed on Whatman No. 1 paper, descending, in isopropanol-water (9:1)[6] and developed by a modification[6] of the method of Trevelyan, Procter and Harrison (15), substituting 5 per cent aqueous sodium thiosulfate for 6 N ammonium hydroxide.

RESULTS

The average values for acid mucopolysaccharides (expressed as hexuronic acid) and 5-hydroxytryptamine recovered from human thrombocytes are recorded in Table I and are compared statistically. The differences noted and their significance are apparent on inspection of the table.

Hexuronic acid levels determined in parallel by the carbazole method and by the orcinol method in acid mucopolysaccharides of human thrombocytes and in various standard acid mucopolysaccharides are recorded in Table II. The carbazole/orcinol ratio of the thrombocyte preparation approached most closely that expected for chondroitin sulfate A or C (16).

Hexosamine and hexuronic acid levels were determined in parallel in the acid mucopolysaccharides from each of two preparations of human thrombocytes. Expressed as μg. per unit of thrombocytes, the comparative levels were 21.1 μg. of hexuronic acid and 30.6 μg. of hexosamine in the first preparation and 12.6 μg. of hexuronic

nificant differences in thrombocyte biuret level observed between Groups B and C on comparison with Group A (see Results).

[4] A full fluorescent spectrum was run on each thrombocyte preparation superimposing this spectrum on that of the serotonin creatinine sulfate standard on an oscilloscopic screen. Identity of spectrum of the thrombocyte preparation with the standard spectrum was always observed.

[5] A commercial chondroitin sulfate (General Biochemicals, Inc.), derived from cartilage and resembling pure chondroitin sulfate A in its paper chromatographic mobility, was similarly hydrolyzed and purified. A commercial glucosamine hydrochloride (Nutritional Biochemicals Corp.) was purified on a Dowex 50 column. A purified galactosamine preparation was made available through the kindness of Dr. Eugene Davidson.

[6] As suggested by Dr. Eugene Davidson.

TABLE I

*Levels of acid mucopolysaccharides (expressed as hexuronic acid) and 5-hydroxytryptamine in human thrombocytes**

Groups	Per thrombocytes from 100 ml. of blood			Per 100 mg. of thrombocyte protein	
	Protein	Hexuronic acid	5-hydroxy-tryptamine	Hexuronic acid	5-hydroxy-tryptamine
	mg.	*μg.*	*μg.*	*μg.*	*μg.*
A) Noninflammatory (average of group±S.D.)	43.8±12.5	45.0±12.9	16.0±8.6	104.7±23.2	38.6±21.9
B) Inflammatory	68.1±24.3	68.6±25.0	12.7±6.7	99.4±19.7	20.0±11.0
t, p (vs. A)	3.567, <0.01	3.207, <0.01	1.069, >0.05	0.654, >0.05	2.543, <0.02
C) Rheumatoid	63.8±22.1	54.3±17.1	10.1±5.5	90.1±27.0	16.9±8.0
t, p (vs. A)	3.170, <0.01	1.661, >0.05	2.147, <0.05	1.501, >0.05	3.387, <0.01
t, p (vs. B)	0.481, >0.05	1.747, >0.05	1.052, >0.05	1.064, >0.05	0.739, >0.05

* Derived from 16 control patients (Group A), 12 nonrheumatoid inflammatory patients (Group B), and 14 rheumatoid patients (Group C).

acid and 27.2 μg. of hexosamine in the second preparation.

From the thrombocyte fraction incubated with testicular hyaluronidase, 4.49 μg. (expressed as hexuronic acid) of nondialyzable acid mucopolysaccharides were recovered. An identical aliquot fraction incubated with buffer yielded 12.99 μg. of hexuronic acid in nondialyzable form and no hexuronic acid could be detected from the hyaluronidase preparation alone.

Paper chromatograms of acid mucopolysaccharides from human thrombocytes are shown in Figure 1.[7] The thrombocyte material showed a mobility quite different from chondroitin sulfate A and somewhat less than that of heparitin sulfate and chondroitin sulfate C. The mobility of the chief component was close to that of chondroitin sulfate B and of the rapid component of heparin. The material did not move as a discrete spot and must be presumed from combined observation of the chromatographic characteristics and the carbazole/orcinol ratio to be a mixture of substances.

Paper chromatograms of hexosamines recovered on hydrolysis of acid mucopolysaccharides from human thrombocytes revealed no trace of glucosamine[8] but did show a well defined galactosamine spot (Figure 2).

[7] All of the spots photographed were metachromatic as developed with toluidine blue except for a dark blue spot at each origin of the thrombocyte fraction.

[8] The method used does not differentiate between glucosamine and mannosamine but does separate both of these substances from galactosamine.

DISCUSSION

The pattern of quantitative recovery of acid mucopolysaccharides from human thrombocytes differed from that of 5-hydroxytryptamine. The acid mucopolysaccharide content tended to vary with the amount of thrombocyte protein present per unit of blood, whereas the 5-hydroxytryptamine content tended toward constancy per unit of blood regardless of the amount of thrombocyte protein present.

The variation in level of acid mucopolysaccharides from human thrombocytes with the amount of thrombocyte protein present per unit of blood suggested that the acid mucopolysaccharides constituted a structural component of

TABLE II

Carbazole/orcinol ratio in acid mucopolysaccharide fraction from human thrombocytes

Material	Hexuronic acid (μg. per sample)		
	Carbazole	Orcinol	Ratio
Acid mucopolysaccharides of human thrombocytes from 100 ml. blood	51.7	55.9	0.925
Commercial chondroitin sulfate (see Experiments) A,* 100 μg.	20.4	17.2	1.186
Chondroitin sulfate B,† 100 μg.	11.7	25.7	0.455
Heparitin sulfate,† 100 μg.	29.6	16.5	1.794

* Run previously by other orcinol methods in parallel with chondroitin sulfate A† to yield carbazole/orcinol ratio identical with that of the standard chondroitin sulfate A.

† Obtained through the kindness of Dr. Karl Meyer.

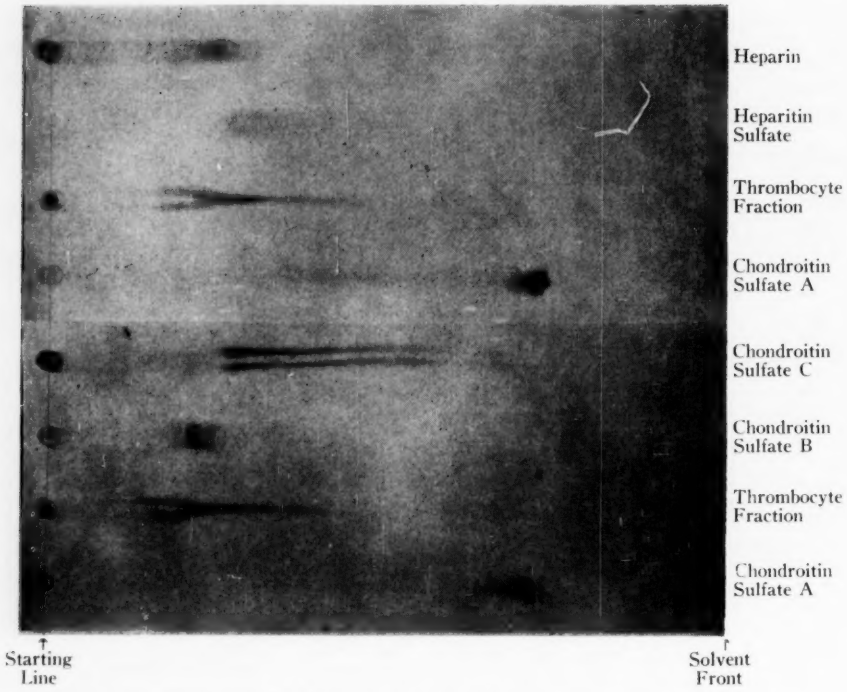

FIG. 1. CHROMATOGRAPHIC COMPARISON OF ACID MUCOPOLYSACCHARIDES FROM HUMAN THROMBOCYTES WITH VARIOUS STANDARDS

Solvent 48 per cent ethanol in pH 6.8, 0.067 M phosphate buffer.

thrombocytes or else were present in thrombocytes by virtue of some homeostatically regulated process that kept the amount present relatively constant. As the amount of thrombocyte protein present per unit of blood from patients with nonrheumatoid or rheumatoid inflammatory processes was increased, so also there was noted an increase in the amount of acid mucopolysaccharides

FIG. 2. CHROMATOGRAPHIC COMPARISON OF HEXOSAMINES OF HYDROLYZED ACID MUCOPOLYSACCHARIDES FROM HUMAN THROMBOCYTES WITH GLUCOSAMINE AND GALACTOSAMINE STANDARDS

Solvent isopropanol-water (9:1).

from thrombocytes per unit of blood. The parallel was close in patients with nonrheumatoid inflammatory disease and in patients with no evidence of inflammatory disease. Patients with rheumatoid arthritis, in contrast, showed a somewhat lower ratio of acid mucoplysaccharides to protein in thrombocytes but this lowering of ratio did not achieve statistical significance.

The 5-hydroxytryptamine content on the other hand and as noted showed no positive correlation with the amount of thrombocyte protein present per unit of blood and was in fact slightly less per unit of blood on comparison of the two inflammatory groups B and C with the noninflammatory control group A, despite the increased amount of thrombocyte protein present in these two groups. This lesser amount of 5-hydroxytryptamine in thrombocytes per unit of blood was of possible significance statistically ($p < 0.05$) only on comparison of the rheumatoid group with the control group. The amount of 5-hydroxytryptamine per unit of thrombocyte protein was significantly less in both of the inflammatory groups as compared to the control group. The overall trend of the observations concerning 5-hydroxytryptamine was thus in accord with the concept established earlier by others (17, 18) that virtually all of the 5-hydroxytryptamine carried in the blood is bound by thrombocytes and with the conclusion that a fairly uniform total amount is apparently present normally for such binding, since the thrombocyte content of 5-hydroxytryptamine normally remains within fairly uniform limits. The slightly lesser total amount observed in the two inflammatory groups then becomes of interest and one could wonder whether the 5-hydroxytryptamine total content is restored to the blood less quickly than are thrombocytes following the extravascular appearance of 5-hydroxytryptamine which Spector and Willoughby (19) have shown occurs early in the evolution of the inflammatory process.

Qualitative studies of the acid mucopolysaccharides of human thrombocytes yielded conflicting data that is best explained by postulating a mixture of substances. The material yielded nothing resembling chondroitin sulfate A on paper chromatography and resembled more in its mobility either chondroitin sulfate B or the rapid component of heparin; the mobility was slightly less than that of heparitin sulfate or of chondroitin sulfate C. However it is apparent from inspection of Figure 1 that the overlap in all standards except chondroitin sulfate A was such that other methods had to be utilized for further characterization of the material. The chromatographic data excluded the likelihood of detectable chondroitin sulfate A and showed the fraction to have metachromatic staining qualities and a mobility consistent with any of the other standards used. Of the five acid mucopolysaccharides used as standards, however, only the three chondroitin sulfates contain galactosamine rather than glucosamine or mannosamine (16, 20, 21). Only galactosamine was detected in the hydrolysate of the acid mucopolysaccharide fraction from human thrombocytes. Thus heparin and heparitin sulfate appeared to be unlikely components of the fraction and interest centered on chondroitin sulfate B and C. Chondroitin sulfate B is resistant to testicular hyaluronidase while chondroitin sulfate C is hydrolyzed by this enzyme preparation (22). The thrombocyte fraction was partially hydrolyzed by testicular hyaluronidase. The carbazole/orcinol ratio of pure chondroitin sulfate B is low while that of pure chondroitin sulfate A or C achieves somewhat more than unity (16). The carbazole/orcinol ratio of the thrombocyte material was slightly less than one and definitely in excess of that of pure chondroitin sulfate B. It would seem probable that the acid mucopolysaccharides of human thrombocytes are a mixture rather than any new substance. A mixture of chondroitin sulfate B and C would best account for all of the data presented here although there is no conclusive evidence for either substance. The acid mucopolysaccharides from human thrombocytes were quite different in their paper chromatographic mobility from the acid mucopolysaccharides previously recovered from human urine and leukocytes (10).

SUMMARY

1. Qualitative characterization of the acid mucopolysaccharides of human thrombocytes has yielded data suggesting a mixture of substances. The data suggest but by no means prove the possibility of a mixture of chondroitin sulfates B and C.

2. No correlation was found between the acid mucopolysaccharide and the 5-hydroxytryptamine content of human thrombocytes. The acid mucopolysaccharide content correlated with the amount of thrombocyte protein present per unit of blood. The 5-hydroxytryptamine content correlated with the amount of blood from which the thrombocytes were separated.

3. On comparison of three groups of patients (noninflammatory, nonrheumatoid inflammatory and active rheumatoid), the thrombocyte protein level per unit of blood was significantly elevated in the inflammatory and rheumatoid groups, as was the acid mucopolysaccharide content of thrombocytes per unit of blood. The 5-hydroxytryptamine content of thrombocytes, in contrast, was slightly lower in the inflammatory and rheumatoid groups (and to a possibly significant degree in the rheumatoid group), despite the higher level of thrombocyte protein per unit of blood.

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RED CELL, PLASMA AND BLOOD VOLUME IN HEALTHY MEN MEASURED BY RADIOCHROMIUM (Cr^{51}) CELL TAGGING AND HEMATOCRIT: INFLUENCE OF AGE, SOMATOTYPE AND HABITS OF PHYSICAL ACTIVITY ON THE VARIANCE AFTER REGRESSION OF VOLUMES TO HEIGHT AND WEIGHT COMBINED *

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Methods employing radioactively tagged red cells have been widely adopted for measurement of blood volume. However, in comparison with the work which has been done with the dye (1–5) and carbon monoxide (6) methods, little attention has been given to the establishment of mean values for men and women or to study of the variance encountered among healthy subjects. Most clinical investigators have collected their own control data, based often on study of relatively few or not entirely healthy subjects (7–13).

A rather large scatter of data around mean prediction values has been found by all workers, regardless of methods employed, when values for whole blood volume (Vwb), red cell volume (Vrbc) and plasma volume (Vpl) are related to body weight, height, or combinations of these measures (1–5, 13, 14). Some of this variation presumably results from differences in body composition, since blood volume correlates both with body density (5, 15) and with lean body mass (16, 17). However, it has not been shown that predictions based on total body mass are less accurate than those based on lean body mass, which requires a separate measurement (15, 16). Consideration of fat thickness and girth measurement, in addition to height and weight, was found by Hicks, Hope, Turnbull and Verel to improve prediction (14). Gregersen and Nickerson (3) classified subjects according to somatotype and found that variance of blood volume per unit of body surface area was reduced to an important degree when dealing with extreme body types but not in the middle ranges. Divergent results have been reported concerning the influence of age and of physical training on blood volume (2, 18–23).

We have used the Cr^{51} method of Sterling and Gray (24, 25) to measure Vrbc and, indirectly from the hematocrit, Vwb and Vpl in 201 healthy prison inmates. Trivariate regression equations have been derived for the plane surfaces which relate the volumes to height and weight and a graphic system for their rapid application to practical problems has been developed. By analysis of individual differences, "residuals," from the mean regression planes, we have studied the effects of the following factors on the variability of the data: age, body build, habits of physical activity and, to a limited extent, seasonal changes.

SUBJECTS

The subjects were selected by careful screening from normally active volunteers. Most of them were white (see Table I for racial and national origins). Information from the prison health records was supplemented by interviews conducted by a physician from our group, by minifilm X-rays of the chest and by laboratory tests which included complete blood count, sedimentation rate, urinalysis, electrocardiogram and a serologic test for syphilis. Volunteers were rejected if the results of these tests were abnormal, if their blood pressures were above 140 mm. Hg systolic or 90 diastolic, or if they had recognizable disease or past history of illness, such as rheumatic fever or tuberculosis, which might be present

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† Research Fellow of the American Heart Association.

TABLE I

Data in chronologic order from experiments on 201 healthy men

Subject*	Age	Height	Weight	Activity class I, II or III	Somatotype			Hematocrit†	Vrbc†
					En	Me	Ec		
	Years	*cm.*	*Kg.*					*%*	*L.*
F. Bl.	29	178	73.5	II	3	4′	3′	43.0	2.02
W. Ru.	25	180	68.0	I	2′	3	4′	43.0	1.96
B. Re.	27	174	64.3					42.8	2.11
A. Wa. (N)	31	173	67.1		1′	5′	3	41.1	1.62
R. Ke.	27	186	66.2	I	2	3	6	44.0	2.03
E. Yo.	25	183	72.5	II				44.2	2.31
J. Ki.	32	171	67.3	II	2	5	2	44.6	1.76
R. No.	29	180	72.5	I	3	4′	3′	47.6	2.16
D. Br.	24	187	76.6	II	3	4	4′	51.2	2.42
R. Cu.	29	180	80.2	I	3	5	2′	48.3	2.49
E. Sm. (N)	38	177	79.2					49.7	2.36
J. Li.	27	173	67.9	I	3	4′	3	44.5	2.01
W. Sm.	34	187	95.7					48.6	2.70
W. Bu.	37	182	66.2	I				46.2	2.01
J. St.	42	173	65.3	I	3	4	4	43.2	1.80
R. Wa.	33	180	71.6	I	2′	4	4′	46.4	2.26
W. Sm.	26	178	77.0		2′	5′	2	48.4	2.30
J. Je. (N)	33	177	87.0	I				46.9	2.37
P. Ki.	26	186	102.0	III	4	5′	1′	47.3	2.54
H. Po. (N)	50	174	110.2‡	II	3′	6′	1	43.6	2.41
R. Ha.	22	169	61.2	I	1′	5′	2	46.0	1.63
R. Su.	30	168	70.3	II				43.0	1.60
R. Sa.	22	183	77.1					45.9	2.35
I. Tu.	25	183	77.1					45.2	1.99
N. McK. (I)	32	183	70.7	II				42.9	2.13
H. Ca.	30	187	86.2	II	3′	4′	3	43.7	2.26
N. St.	33	168	53.9		2′	3	4′	43.4	1.66
D. Ma.	41	182	96.7	II				44.9	2.24
J. He.	35	183	65.7	II				46.5	1.91
G. Al.	43	173	67.6	I	3	4	3	42.6	1.97
W. Be.	26	173	56.6	II				40.1	1.46
M. Va.	21	170	69.3	III	3	5′	2	43.1	1.88
F. Pr.	25	165	58.9					43.2	1.52
M. Re.	27	173	70.3	I	4′	4	3	44.7	1.85
R. Ma.	24	174	63.4	II	3	3′	4	43.3	1.90
T. Ke.	20	183	76.6	I	3′	3′	4′	43.2	2.07
R. Pe. (M)	24	180	64.3	III	2	5	4	41.1	2.18
G. Yo.	34	163	54.9					49.8	2.13
G. Ol.	27	170	61.1	III	2′	4′	3′	43.3	1.79
B. Br.	23	185	85.7	III	3	5	2′	44.0	2.20
K. Kn.	29	180	72.1	I	3′	3′	4′	41.6	2.11
C. Fa.	25	176	65.7	III	2′	5	3	45.6	2.09
B. Ma.	24	171	65.7	III				48.0	2.11
R. Tu.	26	173	71.1	III	3	5	2′	44.2	1.89
J. Fr.	21	183	81.7					43.4	1.97
M. Sw.	31	178	76.2	II	3	4′	3′	48.7	2.08
E. Ri. (M)	35	171	69.2	II	3′	4	3	46.7	2.20
W. Er.	32	180	80.0	II	3′	4′	3′	43.0	2.58
F. Er.	33	171	75.5	II	4	4′	2′	44.4	1.87
S. Ti.	41	163	60.9	III	3	4′	3	47.7	1.55
L. Vi.	36	173	58.0	I	2′	4	4′	42.7	1.63
W. De.	44	170	70.0	III	3	4′	3	43.1	2.08
H. Sa.	26	171	73.0	III	2′	5′	1′	44.6	2.26
K. Sk.	21	182	76.8	II	2′	5	2′	42.7	2.17
B. Ma.	43	182	78.0	III	3′	3′	4	44.7	2.42
R. Bl.	29	175	81.8	III	2	6′	1	44.8	2.07
R. Mo.	31	178	77.3	II	2′	5	2′	46.6	2.26
J. Ca.	30	182	75.0	II	3′	4	4	48.3	2.37
C. Tu.	32	175	68.2	II	3	4	4	43.5	2.04
B. Sy.	35	183	80.0	II	2′	4′	3′	43.5	2.65
D. Hu.	24	185	90.5	II	3	5′	2	45.0	2.72
L. Lo.	35	184	87.3	I	4	3′	3	45.0	2.38
D. Bi.	22	180	87.8	II	4	5′	2	45.6	2.32

* (C) Chinese, (F) Filipino, (I) North American Indian, (M) Mexican, (N) Negro.
† Uncorrected for trapped plasma.
‡ Weight: height ratio exceptionally high. See Figures 1 and 4.

TABLE I—*Continued*

Subject*	Age	Height	Weight	Activity class I, II or III	Somatotype			Hematocrit†	Vrbc†
					En	Me	Ec		
	years	*cm.*	*Kg.*					%	*L.*
R. Al.	27	175	63.6	III	2′	3′	4	47.8	1.95
J. Da.	30	173	75.5	II	2′	5′	1′	46.1	2.33
L. An.	25	175	82.1	III	2	6′	1	49.2	2.17
W. Me.	25	192	84.6	III	2′	4	4	47.2	2.44
W. Co.	30	177	75.0	III	2	6	1′	43.4	1.86
P. Re.	29	179	62.3	I	2	3′	5	40.8	1.67
D. Sm.	21	187	71.0	II	2′	3′	4	42.3	2.08
F. Sh.	26	165	63.2	I	2′	5′	2	48.9	1.80
H. Fr.	37	167	70.0	II	3	5	2	48.8	1.81
J. Ve.	33	170	63.6	III	2	5	3	45.3	1.90
M. Wi. (N)	30	175	65.0	II	1′	5	4	45.7	1.87
W. McC. (N)	27	191	98.2	III	2′	6	2	43.3	2.58
P. Do.	32	175	71.0	III	2′	5	3	44.9	1.78
R. Wh.	26	180	77.3	III	4	4	3′	42.4	1.73
P. Ca. (M)	26	168	65.5	II	3′	4′	2′	48.4	1.93
F. Pe.	26	176	71.4	II	2′	5	2′	45.2	2.30
R. In.	33	175	71.0	III	3	4	3	42.5	1.75
C. Mo.	23	180	66.0	II	2′	3′	4′	45.4	1.84
J. Po.	45	163	63.6	I	3′	5	2	43.0	1.61
W. Tw.	22	185	78.2	III	3′	4	4	45.5	2.13
R. Ga.	32	177	81.4	I	4	4	2	40.2	1.83
L. Ba.	31	178	72.3	I	3	4′	4	44.6	1.95
B. Ho.	26	173	64.1	II	1′	5′	3	41.6	1.58
R. Bl. (M)	27	177	65.5	II	3	4	4′	48.1	1.93
H. Pi. (I)	22	177	70.9	I	3	5	3	44.2	1.82
S. Ro.	30	179	60.5	I	2	2′	5′	39.4	1.44
W. Ti. (N)	27	164	64.6	I	2	6	1′	48.4	1.95
M. Wa.	33	182	78.2	II	3′	3′	3′	47.6	2.03
E. Ch.	29	175	75.6	II	3	5	2	42.1	1.84
M. Pa. (F)	27	180	93.2	III	4	5′	2	52.8	2.69
C. Ho.	21	180	67.3	I	3′	3′	4	46.3	1.87
F. McG.	26	180	66.9	I	2′	2′	5	46.5	2.00
J. McC.	26	183	66.8	III	3	2′	5′	48.5	2.07
G. Lo. (C)	30	170	54.1	I	2′	2′	5′	47.2	1.72
C. Fi.	31	157	55.5	II	3	5	2′	48.2	1.54
E. Fl.	30	183	82.8	II	3	4′	3′	48.4	2.21
A. Ow.	28	179	86.8	III	3	6	2	44.9	2.13
P. Ca.	26	163	59.6	I	3	5	2	47.3	1.89
P. Li.	35	168	68.6	I	4	4	2′	45.9	1.70
E. Re. (N)	22	183	74.2	II	1′	5′	3′	42.6	1.91
E. Mu.	40	183	74.2	I	2′	3′	4′	46.3	2.32
R. Mi.	33	171	76.4	I	3′	5	2′	46.5	1.65
E. McN.	25	174	55.9	II	2	2′	5	43.6	1.82
J. Au.	26	179	80.9	II	4	4′	3	44.2	2.07
W. Jo.	26	175	59.2	I	2′	2′	5	45.5	1.53
J. Co.	27	177	85.9	II	4′	5	2′	54.0	2.62
R. Mo. (N)	27	180	70.0	II	2	4′	4	43.5	1.99
F. Sm.	35	166	69.6	I	3	5	2	41.9	1.60
J. Ja. (N)	32	171	69.2	I	2	5′	2′	53.5	2.15
P. Ca.	24	170	66.4	II				43.4	1.74
F. La.	34	188	81.0	II	2	4′	4	45.9	2.45
G. Fi.	24	175	80.5	I	4	5′	2	45.3	2.12
B. Fa.	27	179	68.2	II	2′	3′	4	44.0	1.79
C. Bi.	21	165	65.5	II	3	5′	1′	50.0	2.25
G. Tr.	29	180	91.0	III	4	5	2	44.9	2.24
A. Ko.	35	179	61.8	III	1′	4	5	40.7	1.63
H. Or. (N)	40	179	112.7‡	I	5′	4′	1′	53.0	3.02
R. Le.	37	170	74.1	II	3	5	2	46.7	2.03
T. Ke.	30	170	73.2	III	2′	6	2	46.2	2.05
V. Sl. (N)	32	173	74.2	II	3′	4′	2′	46.6	2.23
A. Si.	43	163	79.6	I	4	5	1	45.9	2.21
R. Ca.	36	175	70.5	II				44.8	1.94
J. Sm.	31	171	84.2	III	4′	4′	1′	44.3	2.18
W. Mo.	35	173	68.2	III	3	4′	3′	44.3	1.78
M. Du.	25	178	66.0	III	1	5′	3′	41.7	2.04
J. Ha.	45	171	63.7	I				40.5	1.66
J. Al.	27	184	71.8	I	2′	3′	4′	45.5	2.47
L. En.	29	173	67.3	I	2′	5	3	44.9	1.83

TABLE I—*Continued*

Subject*	Age	Height	Weight	Activity class I, II or III	Somatotype			Hematocrit†	Vrbc†
					En	Me	Ec		
	years	*cm.*	*Kg.*					*%*	*L.*
R. Gu.	26	163	78.7	III	3	7	1	46.9	2.14
W. Ki.	28	170	61.8	I				48.5	1.72
H. Co.	30	185	72.8	III	2′	3′	5	49.1	2.35
H. We.	21	173	66.8	I	3′	4′	3	48.8	1.72
A. Ma.	28	168	65.4	II	3′	4′	2′	47.6	2.05
R. Ba.	22	178	73.2	III	3	5	3	48.5	2.22
R. Bo.	25	180	63.2	I	2′	2′	5′	42.9	1.73
E. Ro.	27	173	63.7	II	3	4	4	46.5	1.78
R. Mo.	28	174	69.6	II	3	5	2′	43.9	1.88
W. Re.	37	178	95.5	II	5	4	3	44.9	2.43
T. La.	25	170	82.8	II	5	5	1′	45.2	1.98
J. Ri.	48	179	67.3	II	2	4	5	44.9	1.82
D. Hu.	19	178	64.1	II	2′	4	4′	43.1	1.73
V. Fl.	22	173	65.5	II	3	5	4	45.6	1.89
E. Ge.	30	170	63.2	I	3	4	3	42.8	1.73
E. Zu.	39	177	77.3	II	3	4′	3	48.6	2.12
A. Ma.	39	184	78.2	III	3′	3	4′	46.7	2.19
J. St.	24	180	64.6	I	2′	3′	5	44.6	1.80
D. Jo. (N)	26	178	70.0	II	1′	5′	3′	43.5	1.84
W. Ka.	23	184	74.1	I	3	4	4	39.8	1.91
R. Or.	22	170	72.8	III	3′	5′	2	48.4	2.10
R. Ma.	23	177	74.6	I	3	5	2′	44.8	2.11
W. Co.	28	170	58.2	I	2	4	4	45.0	1.90
R. Cu.	22	173	61.8	II	3	4	4	42.2	1.96
J. Ad.	27	183	74.1	II	2′	4′	4	42.0	2.28
D. Ra.	39	184	70.9	I	2	3′	5	43.9	2.01
G. Lo.	20	180	80.9	II	4	5	2	43.3	2.21
C. Ba.	21	178	66.4	II	2	4′	4	42.8	1.81
W. Th.	42	171	66.8	I	2′	4′	3′	40.9	1.87
J. An.	28	185	87.3	III	1′	6′	2′	45.8	2.48
R. McA.	25	175	67.3	II	2′	4′	3	43.5	2.07
C. Mo.	25	171	65.0	I	2′	5	2′	45.9	1.94
C. Fa.	29	165	71.0	III	2	6′	1	45.3	2.05
R. Sm.	37	170	64.1	II	2′	4′	2′	46.9	1.92
G. Br.	27	174	75.0	III	2	6	2	47.7	2.15
C. Fi.	29	180	81.4	I	3′	4′	2′	44.9	2.13
B. Kl.	25	175	83.7	II	5	4	2	45.0	2.23
C. Sl.	24	177	78.7	III				51.2	2.64
F. Sp.	30	184	75.5	I	3	4	4′	47.5	2.23
J. Sa.	29	169	66.8	II	3′	5	3	49.5	1.90
H. Wi. (N)	24	169	60.5	III	2	5	3′	40.3	1.60
R. Fa.	24	163	60.5	II				44.1	1.35
J. Ja.	24	180	69.1	III	2	4′	4	42.3	1.74
W. Jo.	33	174	68.2	I	3	4	3′	44.8	1.91
H. Co.	52	188	73.2	II	2	3	5	42.9	1.73
J. Jo.	28	191	66.4	I	1′	1′	6′	43.9	1.98
W. Sc.	25	177	66.8	I	3′	3′	3′	46.4	1.88
J. Ar.	27	178	66.4	II	2′	3′	4	45.2	2.07
J. St.	24	180	64.6	I	2	4	5	44.7	2.29
T. Pr.	36	189	90.5	III	2	5′	3	45.1	2.58
J. Me.	33	168	58.2	II	2	5	3	46.0	1.82
M. Bu.	23	173	63.6	II	2′	4	4	42.0	1.78
F. Br.	36	170	65.5	I	3	4	3	49.0	2.18
E. Ru.	29	169	62.3	II	2′	4′	3	45.2	1.81
R. Kr.	24	179	67.8	I	2	4′	4	44.0	1.95
D. Co.	28	173	62.3	II	1′	5	3′	46.1	1.94
R. Ce.	33	174	65.0	II	2	4′	3′	43.0	1.82
K. O'N.	23	183	86.8	II	4′	4′	2′	44.3	1.92
M. Si.	34	173	67.3	III	2	5	3	44.9	2.26
K. Kr.	25	188	83.7	I	2	5′	3	48.5	2.71
R. Cl.	30	182	80.4	II	2	5′	2′	47.6	2.47
S. DeR.	22	183	71.4	II	2	5	3′	43.2	2.15
D. La.	27	189	72.3	II	2	3	5′	44.9	2.16
J. Ca.	29	179	72.8	I	2	5	3	42.5	1.80
W. Sh.	32	173	66.4	I	2′	4′	3	41.5	1.59
R. Ro.	33	183	80.5	II	2	5′	3	45.1	2.36
W. Ch.	26	175	69.6	II	1	6	2′	44.2	2.14
S. Ga.	42	180	65.5	I				45.9	1.97
M. Br.	29	183	73.7	I	2′	4	4	47.1	2.09
C. We.	37	172	72.3	I	2′	5	3	46.1	2.08

in latent or chronic form. No cases of gross clinical obesity were included. The men had stayed in San Quentin State Prison, where there is little seasonal variation of outdoor climate, for various periods of time. None had stayed recently at high altitudes or in climates of extreme heat or cold. The series included 46 men who had donated blood once and seven who had donated blood twice in the year preceding the experiment. Donors were not accepted if they had given blood within 42 days; those who had donated twice were not accepted within 60 days of the last bleeding. The basis for the decision to include blood donors in the series of "normals" will be shown under Results.

PROCEDURE AND METHODS

On the day before the experiment, blood was taken for tagging and the laboratory screening tests were performed. The subject stayed in the prison hospital overnight and received no food or medication after retiring in the evening. He was also requested not to smoke. On the morning of the experiment, he reclined for at least 30 minutes before injection of the tagged cells and until the final sample for measurement of blood volume had been taken. Wintrobe hematocrit tubes were filled in duplicate from each blood sample within three hours of collection and centrifuged for 30 minutes at 3,000 rpm (radius 15 cm.). The height of the cell column was read to the top of the buffy coat, and no correction was applied for "trapped plasma."

The volume of cells (Vrbc) was measured by a modification of the Cr^{51} method of Sterling and Gray (24) which we have described elsewhere (25) and which will be analyzed in a forthcoming publication (26). Essentially, the procedure was as follows: *1*) About 15 ml. of the subject's blood was tagged with 100 to 200 μc. of $Na_2Cr^{51}O_4$, containing 0.1 to 1.0 μg. of Na_2CrO_4 per μc. The erythrocytes were then washed and resuspended in saline and stored overnight in the refrigerator.[1] *2*) Ten ml. of the cell suspension was delivered from a calibrated syringe and washed quantitatively into the subject's vein with not more than 30 ml. of saline, using an indwelling needle and a small infusion system. *3*) Two or three samples of blood were taken from the same needle beginning not less than 20 minutes after delivery of the tagged cells. The reported data are based on the averages of the derived values. A very slow infusion of saline provided for patency of the needle, but this was discontinued and at least 2 ml. of blood was taken from the vein and discarded before collection of any sample. *4*) Vwb was determined by comparing the radioactivity of each blood sample with that of the tagged cell suspension. Vrbc was derived by multiplying Vwb and the hematocrit of the same blood sample. Vpl was obtained by subtracting Vrbc from Vwb. No factor was introduced to correct the data for the probable difference between the hematocrit of venous blood and that of the body as a whole (29–31).

On the day of the experiment pulse and blood pressure were measured. The subject's height and weight in the nude were measured to the nearest half inch and pound, respectively, and he was photographed as prescribed by Sheldon (32) for determination of somatotype. His age to the nearest birthday was recorded and the medical interview was completed.

On the basis of the medical interview, supplemented by information from associates and supervisors, each subject was assigned to a physical activity group. Group I (light) included clerical workers, laboratory assistants, students and unemployed persons who did not participate in sports. Group II (moderate) consisted of janitors, garden and yard workers, messengers, cooks, electricians, and so forth. Group III (heavy) consisted of men who worked as plumbers, roofers, plasterers, laundry helpers and quarry and construction laborers. Because of activity in sports, a person who by occupation belonged in Group I might be placed in Group II. If he participated intensively in strenuous sports, such as weight-lifting, football, wrestling or boxing, a person whose work was light or moderate might be placed in Group III.

Each subject was classified[2] according to a scale of 13 (seven whole grades and six half grades, shown in Table I as primes) for each of the three somatotype components, endomorphy (obesity), mesomorphy (muscularity) and ectomorphy (linearity).

RESULTS

1. Volumes in relation to height and weight

Table II lists the mean values for Vrbc, Vpl and Vwb, with the regression equations describing the relationships of the volumes to the body measurements. The regression lines and planes were fitted by the method of least squares. For Vrbc, Vpl and Vwb, the coefficient of variation is smaller if the volumes are related to height and weight combined or to the calculated body surface area (Du Bois' formula), than if they are related to height or weight alone. The values for whole blood have a smaller coefficient of variation than those of the two components. The bivariate equations represent straight lines and the trivariate equations, planes without curvature.

In Figure 1, each of the 201 subjects is plotted

[1] Overnight storage of the cells makes it possible to inject them into the fasting subject at a convenient time before breakfast. *In vitro* tests and animal experiments have shown that handling the blood in this way does not affect the accuracy of the method (27, 28).

[2] We are greatly indebted to Dr. C. W. Dupertuis of Western Reserve University, who, at our request, kindly agreed to make an independent determination of somatotypes. No classification was considered correct unless approved by Dr. Dupertuis.

TABLE II

*Regression equations of Vrbc, Vpl and Vwb to weight alone, height alone, weight and height combined and to body surface area**

	Mean of all values	Predicted values	Standard deviation	Coefficient of variation
	ml.	*ml.*	*ml.*	*%*
Vrbc	2,030		290	14.2
		1) 22.4 × height − 1,930	250	12.2
		2) 21.4 × weight + 490	200	9.7
		3) 8.6 × height + 18.6 × weight − 830	190	9.4
		4) 1,550 × surface area − 890	190	9.4
Vpl	2,460		330	13.2
		5) 29.7 × height − 2,770	260	10.7
		6) 19.6 × weight + 1,050	260	10.7
		7) 19.9 × height + 13.1 × weight − 2,000	240	9.7
		8) 1,580 × surface area − 520	240	9.8
Vwb	4,490		570	12.6
		9) 52.1 × height − 4,700	450	10.1
		10) 41.0 × weight + 1,530	400	8.9
		11) 28.5 × height + 31.6 × weight − 2,820	370	8.1
		12) 3,140 × surface area − 1,410	360	8.1

* Height in cm.; weight in Kg.; surface area in square meters as calculated from Du Bois' formula. Values are uncorrected for trapped plasma and for differences between body hematocrit and venous hematocrit.

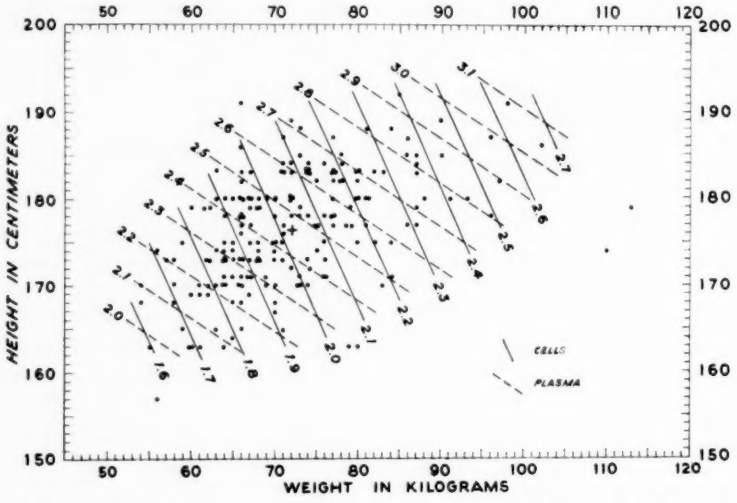

FIG. 1. RED CELL AND PLASMA VOLUMES IN RELATION TO HEIGHT AND WEIGHT

Each solid circle represents one subject, plotted according to his height and weight. The average of the heights and weights is shown by +. The solid and dashed contour lines give the mean predicted Vrbc and Vpl, respectively, to the nearest 0.1 L., calculated from Equations 3 and 7, Table II. The mean predicted volumes of a man 175 cm. tall and weighing 70 Kg. are: Vrbc = 1.98 L., Vpl = 2.40 L., and Vwb = 4.38 L. Values are uncorrected for trapped plasma and for differences between body hematocrit and venous hematocrit. Vrbc includes leukocytes and platelets.

The contour lines are constructed by introducing the successive mean values for Vrbc and Vpl in their respective regression equations (No. 3 and No. 7, Table II) and computing the height for a given weight or vice versa.

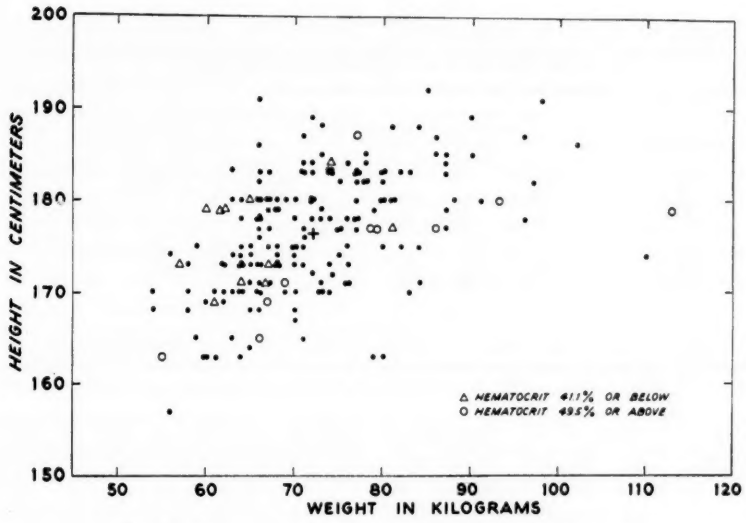

FIG. 2. RELATION OF HEIGHT AND WEIGHT TO HEMATOCRIT

Each subject is plotted according to height and weight as in Figure 1. The 11 men with the lowest hematocrits, 41.1 per cent or below and represented by △, appear mostly in the left upper area. The 10 men with the highest hematocrits, 49.5 per cent or above and represented by ○, appear mostly in the right lower area.

according to his height and weight. The location of the regression plane for Vrbc in relation to combined height and weight (Equation 3, Table II) is represented by the solid contour lines progressing from the left lower to the right upper corner of the chart. The regression plane for Vpl (Equation 7, Table II) is represented by the dotted contour lines. The contour lines for Vrbc are steeper than those for Vpl, indicating that Vrbc is relatively more dependent on weight than height, whereas the reverse is true of Vpl. Although the differences are small, the hematocrits of short, heavy men tend to be slightly higher than those of tall, thin men, as shown in Figure 2.

2. Influence of factors other than height and weight as shown by analysis of "residuals"

Analysis of the differences between the observed volumes in each subject and the average volumes for his height and weight, *i.e.*, "residuals," provided a means of testing the influence on blood volume of factors other than weight and height when considered independently. The predicted Vrbc and Vpl of each subject were calculated, using Equations 3 and 7, Table II. Each subject's residual Vrbc and Vpl were then found by subtracting his predicted from his observed volumes.

A positive correlation was found between residuals for Vrbc and Vpl ($r = +0.42$).

A) Age. Table III shows the distribution of ages and the mean residuals for Vrbc and Vpl of the subjects within each five year age group. The largest number of men were in their third and fourth decades; a few were over 40. Comparison of mean residuals with their standard errors indicates that age has little effect, except on the Vrbc of subjects in the highest age group.

TABLE III

Influence of age on Vrbc and Vpl

Age	Number of men	Residuals Vrbc		Residuals Vpl	
		Mean	S. E.*	Mean	S. E.*
years		*ml.*	*ml.*	*ml.*	*ml.*
19–24	42	−18	29	14	37
25–29	76	15	22	−5	27
30–34	45	4	28	−11	35
35–39	22	10	41	−22	51
40–44	11	40	57	88	72
45–52	5	−236	85	−42	106

* Standard error.

TABLE IV

*Classification of 177 men with respect to endormorphy, mesomorphy and ectomorphy and mean residuals in ml. of Vrbc and Vpl of grouped classes**†*

Endomorphy	Mesomorphy	Ectomorphy 1, 2			Ectomorphy 3, 4, 5			Ectomorphy 6, 7		
	1, 2				(6)	**−97**	*−148*	(1)	**−60**	*−150*
1, 2	3, 4, 5	(13)	**116**	*19*	(59)	**7**	*62*	(1)	**30**	*10*
	6, 7	(10)	**26**	*47*						
	1, 2				(1)	**80**	*−330*			
3, 4, 5	3, 4, 5	(37)	**−21**	*−33*	(46)	**−10**	*−27*			
	6,7	(3)	**−133**	*80*						
6,7										

* Number of individuals in parentheses; mean Vrbc residual in **bold** type; mean Vpl residual in *italic* type.
† The calculated linear regression equations of residuals on the three somatotype variables are as follows: *1)* Expected Vrbc residual (*ml.*) = − 27En + 1Me − 17Ec + 114; *2)* Expected Vpl residual (*ml.*) = − 38En + 25Me − 8Ec + 19.

B) Somatotype. A total of 177 individuals were somatotyped. They fell into 43 distinct groups when a scale of one to seven was used to describe each component (endomorphy, mesomorphy, ectomorphy). Analysis of residuals showed that the reduction in variance which results from considering somatotype when height and weight are fixed is numerically small and of doubtful statistical significance (p for Vrbc = 0.08, for Vpl = 0.23).

In Table IV, the degrees of endomorphy, mesomorphy and ectomorphy have been reduced even further by grouping together the less extreme classes (three, four and five) and the high and low extremes (one and two; six and seven). Thus, the number of combinations encountered in our material is reduced to 10 and the number of individuals per group is larger. Inspection of the table shows the predominance of muscularity in our subjects. The mean residuals show no strong trends, although there appears to be a tendency toward negative residuals in men of low muscularity, positive residuals in men of high muscularity, low linearity and low obesity and negative residuals in men of high muscularity and low linearity but moderate obesity.

TABLE V

Influence of physical activity on Vrbc and Vpl

Physical activity class	Number of men	Residuals Vrbc Mean	Residuals Vrbc S. E.*	Residuals Vpl Mean	Residuals Vpl S. E.*
		ml.	*ml.*	*ml.*	*ml.*
I	65	−19	24	−37	30
II	80	1	21	17	27
III	45	15	29	22	36

* Standard error.

C) Physical activity. Table V shows the fairly uniform distribution among Groups I, II and III of the 190 subjects who were classified according to physical activity. The residuals of both Vrbc and Vpl appear to increase with increasing physical activity but the effect is slight after allowances for weight and height. The differences are not statistically significant (for Vrbc, $p = 30$; for Vpl, $p = 0.15$).

D) Seasonal and within-month variations. Data were not collected primarily for evaluation of these factors. However, certain trends during the 25 months of study suggest that seasonal fluctuations of blood volume may occur even in the relatively uniform climate of San Quentin. For example, in 1954 and 1955, the mean Vrbc residual for the 30 observations between July 7 and November 9 was + 117 ml. (S.E. 35), and for the 51 observations between November 16 and February 8, it was − 70 ml. (S. E. 27). Mean residuals for plasma are similar, + 156 and − 130, respectively. It should be kept in mind that these two time intervals were selected from the entire 25 month period because they showed the most extreme deviations, suggesting a seasonal effect. By grouping the residuals according to the months in which observations were made, it was found that the variance within months was 15 per cent lower than the variance of all 201 residuals ($p = 0.001$). The relative uniformity of results within months, in contrast to the fluctuations during the entire study period, might be attributed to *a)* seasonal effects, *b)* the types of subjects encoun-

tered, or *c*) methodologic factors. Influence of the latter seems unlikely since the within-month effect was as great at the end of the study as at the beginning and since the personnel, procedures and methods were unchanged throughout.

E) *Blood donations.* Fifty-three blood donors were included in the series. Their residuals of Vrbc and Vpl are plotted in Figure 3 according to the interval of time between donation and experiment. The points are distributed evenly around the zero line which represents the predicted Vrbc or Vpl of each individual calculated from his height and weight. Most of the residuals were within plus or minus one standard deviation of the mean predicted volume so that the variation is no greater in this group than in the population as a whole. The residuals showed no tendency to increase or decrease progressively with time, between 42 and 180 days. For all donors, the mean residuals (ml.) were as follows: Vrbc = + 17 (S.E. 27), Vpl = − 17 (S.E. 33), Vwb = + 0.2 (S.E. 50).

3. *Repeat determinations*

In 15 men the blood volume was re-estimated one or more times after intervals of three to 31 weeks. The mean of the 20 differences for Vrbc was 73 ml. (range, 10 to 160 ml.) and for Vwb, 222 ml. (range, 10 to 710 ml.). These repeat measurements enable us to estimate the amount of variation due to measurement errors and short-term temporal changes. The estimated standard deviation of such variation was 64 ml. for Vrbc and 211 ml. for Vwb. The standard deviations about the regression planes described by Equations 3 and 11, Table II, are reduced only modestly by taking into account the degree of variance shown by the repeat measurements, which includes the errors of measurement and the changes in blood volume of individual subjects from time to time.[3] Thus, for Vrbc almost all of the predictive error seems to relate to intrinsic differences between subjects rather than to errors of measurement or temporal changes within subjects. For Vwb this is so to a lesser degree; this volume is either less stable or less accurately measured, or both.

DICUSSION

Only the observed venous hematocrit values were used in our calculations. It is recognized that the true Vrbc is slightly smaller than that calculated from the Cr^{51} space and observed hematocrit because of plasma trapping in the hematocrit

[3] The S.D. for Vrbc (Equation 3, Table II) is reduced from 190 ml. to $\sqrt{(190)^2 - (64)^2} = 179$ ml. and the S.D. for Vwb (Equation 11, Table II) is reduced from 370 ml. to $\sqrt{(370)^2 - (211)^2} = 304$ ml.

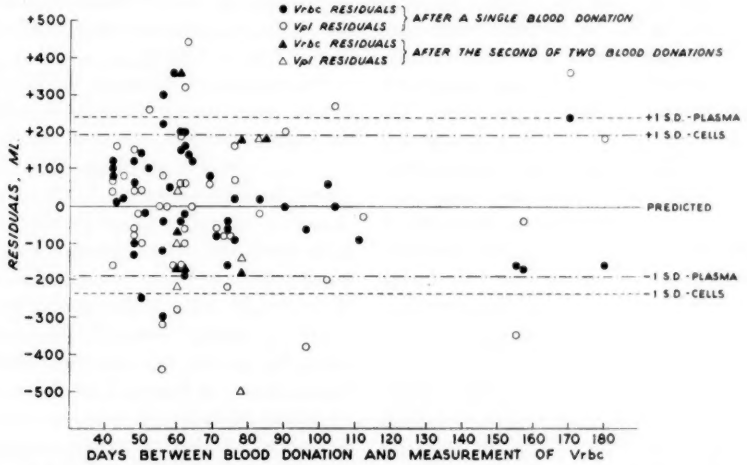

FIG. 3. RESIDUALS OF VRBC AND VPL FOR 46 MEN WHO HAD GIVEN ONE BLOOD DONATION AND SEVEN WHO HAD DONATED TWICE, ARRANGED ACCORDING TO THE TIME INTERVAL BETWEEN THE LAST DONATION AND THE BLOOD VOLUME MEASUREMENT

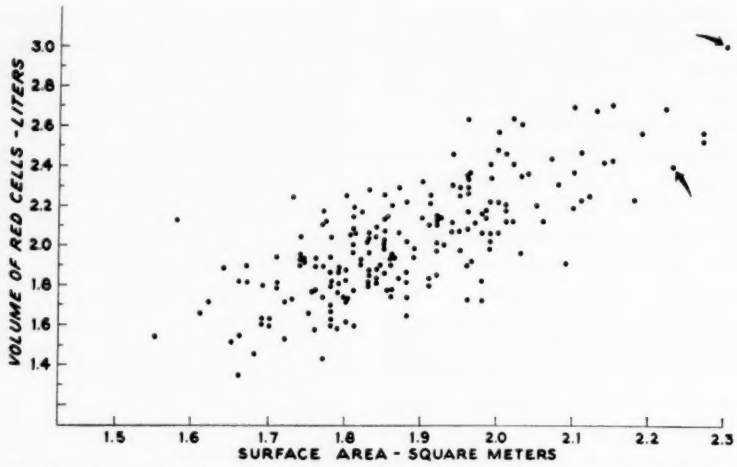

FIG. 4. VRBC OF 201 HEALTHY MEN IN RELATION TO BODY SURFACE AREA AS CALCULATED FROM DU BOIS' FORMULA

The linear regression Equation 4 (Table II) describes the data. Arrows point to two subjects whose weight: height ratios were exceptionally high and who appear to the far right of the population scatter shown in Figures 1 and 2. Data relating to these two subjects are marked by ‡ in Table I.

tube (33) and that Vwb and Vpl may be larger than they appear when calculated from Vrbc and a large vessel hematocrit (29, 30). Application of correction factors such as are used widely at present, 0.96 for trapping (34) and 0.91 for body: venous hematocrit ratio (31), would change the observed values only systematically. Correction factors which apply to data on healthy subjects do not necessarily apply to subjects with disease. Plasma trapping varies with the hematocrit level (35) and the body: venous hematocrit ratio is altered in some circulatory disorders (11, 35), with splenomegaly (36) and in pregnancy (37). Other investigators may conveniently compare their data with ours before they apply any desired correction factors. Vrbc as determined here is independent of the body: venous hematocrit ratio, but Vpl and Vwb are not. It should be noted that Vrbc as reported here includes leukocytes and platelets because hematocrits were read to the top of the buffy coat.

The finding that blood volume relates more closely to one or another combination of height and weight than to either measure alone is in accord with the results of several other studies (1–6, 13, 14). von Porat (4) proposed the use of $(\text{height})^3$ as a reference and Allen and co-workers (5) found that regression to $(\text{height})^3$ and weight gave the most adequate expression of their own and other published data. However, the function, $(\text{height})^3$, is nearly linear over the narrow range of heights encountered among adult men. The predicted Vwb is altered only 28 ml. at most by substitution of height for $(\text{height})^3$ in the equation of Allen and co-workers (5) over the range of heights encountered by these authors (1.57 to 1.89 M.).

The regression equations presented here should not be relied on for heights and weights outside the range covered by our observations, since there is no reason to suppose that the regression planes would continue to be the same beyond the observed domain. Furthermore, the accuracy with which the planes are estimated decreases as we depart from the center (indicated by a cross) of the height: weight scatter diagram (Figure 1).

For predicting "normal" volumes in individual cases, the graphic representation of the regression planes shown in Figure 1 has several advantages: *a*) No calculations are required; *b*) one can see at a glance whether the individual's height and weight are within the range covered by our calculations; and *c*) any existing peculiarities of height: weight relations of the individual under

consideration are revealed. That these may be obscured when calculated body surface area is used as the basis of prediction was pointed out many years ago by Rowntree and Brown (1) and by Gibson and Evans (2). For example, two heavy men are found to the far right of Figure 1. Although noticeably different from the rest of the population when located on the scatter diagram, they cannot be distinguished from the other large individuals in our series when the data for Vrbc are plotted against body surface area, as shown in Figure 4.

Regression of Vwb to height and weight accounted for only 56 per cent of the original variability of our data. The coefficient of variation (8.1 per cent) after this regression is of about the same magnitude as has been found in other studies after regression or correlation of blood volume to weight and height or to surface area. The magnitude of the coefficient depends on the homogeneity and size of the population under investigation. It was comparatively low in a group of average American medical students and considerably higher than ours in a group of subjects selected for extremes of somatotype (3). Our sample of adult males was of intermediate homogeneity, in that it included men from several racial origins whose ages extended to above 50 years and included physically active as well as sedentary persons, but only a few examples of extremely linear or obese body build. Most of the variation to be accounted for after consideration of height and weight is biologic rather than methodologic, as shown by our repeat studies and by similar experiences reported by workers using a variety of methods, for example, P^{32} (7, 12, 13), Cr^{51} (10), I^{131} (15), and T-1824 (5, 13, 16).

Within the population available to us for study, we have found that age, somatotype and habits of physical activity influence Vrbc and Vwb only slightly when considered independently from height and weight. This may be because each of these characteristics is strongly linked in its effect on blood volume with body size and composition. The effect of each characteristic is of course minimized when examined by analysis of residuals about the height × weight regression planes. Available evidence suggests that individual differences with respect to the content of fat and lean tissue in the body have only a modest effect on blood volume. This is probably because the blood content of fat tissue is not insignificant (5, 15). Hicks, Hope, Turnbull and Verel (14) found that consideration of fat thickness and girth in addition to height and weight lowered the coefficient of variation for Vwb from 9.6 to 7.6 per cent. Vwb, Vrbc and Vpl have been found to be directly related to body density (5, 15), and Muldowney (17) found a surprisingly good correlation between Vrbc and lean body mass, considering that both were determined indirectly. On the other hand, Inkley, Brooks and Kreiger (16) found no better correlation between Vpl and lean body mass, determined by densitometry or by antipyrine space, than between Vpl and total body mass, and Huff and Feller (15) were unable to improve the prediction of blood volume by determining body density and calculating the expected blood content of fat and lean tissues separately.

The data of Gibson and Evans (2) appeared to show that blood volume per unit body size was less in large than in small subjects, so that the curves relating volumes to height, weight and body surface area were flattened at the top. We have been unable to corroborate this finding; none of our regression lines or planes show evidence of curvature. The only apparent explanation for the difference is that our large subjects were relatively more muscular and less obese than theirs. In most other respects, our results are in remarkably good agreement with those of the early workers who used the dye methods. The positive correlation found between residuals for Vrbc and Vpl ($r = +0.42$) means that a man whose Vrbc is larger than average for his height and weight will also tend to have a large Vpl. This is in contrast to the inverse relation between Vrbc and Vpl found in certain pathologic states, *e.g.*, anemia and polycythemia (38), and suggests that the normal biologic determinants of blood volume may be concerned with circulatory volume rather than with total circulating hemoglobin.

The apparent absence of age effect in the third to fifth decades is in agreement with several reports (6, 8, 13). The suggested tendency for the volumes per unit size to decrease in the sixth decade corroborates the findings of Gibson and Evans (2) and of Baker, Kozoll and Meyer (20). The question of age, however, requires further

study, inasmuch as both the above-mentioned studies (2, 20) and our own involved few subjects over 50 and since discordant results were found in two investigations particularly devoted to the problem of aging (18, 19).

We have found that muscularity (mesomorphy) has a slight positive effect, while both linearity (ectomorphy) and obesity (endomorphy) have negative effects on the residuals about the height × weight regression planes. Also, a slight progressive positivity of residuals was found with increasing grades of habitual physical activity. This supports Sjöstrand's hypothesis (39) that Vrbc is primarily dependent on total muscle mass and, equally well, the thesis that blood volume may be more dependent on lean than on total body mass. In contrast, Bass, Buskirk, Iampietro and Mager (22) found no increase in Vpl or Vrbc after three weeks of vigorous physical conditioning and Buskirk (23) was unable to demonstrate differences in Vpl or Vwb per unit of total or fat-free body weight in moderately or rigorously trained athletes and nonathletes. The demonstration by Kjellberg, Rudhe and Sjöstrand (21) of large differences between the blood volumes of athletically trained and untrained persons requires re-examination. The carbon monoxide method which they used is particularly unsuitable for this problem, since 15 per cent of the test dose of gas leaves the blood during the measurement and goes mostly to muscle pigments (25, 27).

SUMMARY

1. A group of 201 men were screened for health and classified as to age, habits of physical activity and somatotype. The red cell volumes (Vrbc) of these subjects were measured with Cr^{51} tagged cells; plasma (Vpl) and whole blood volumes were derived indirectly from venous hematocrits.

2. Regression equations were derived, expressing the relations of the volumes to body weight and height and to combinations of these measures. From these data a chart was devised from which the expected Vrbc and Vpl for a man of given height and weight can be found conveniently.

3. In the sample of population examined, age, elements of the somatotype and habits of physical activity were found to influence the variance of the data only slightly, after effects of height and weight had been accounted for.

ACKNOWLEDGMENT

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STUDIES OF ANTIRICKETIC ACTIVITY IN SERA FROM PATIENTS WITH DISORDERS OF CALCIUM METABOLISM AND PRELIMINARY OBSERVATIONS ON THE MODE OF TRANSPORT OF VITAMIN D IN HUMAN SERUM *

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Proof of the curative action of vitamin D for rickets was established by the studies of many investigators (1, 2). It is now known that therapeutic doses of this vitamin bring about increased calcium absorption from the intestinal tract (3), while massive doses induce hypercalcemia (4) and, experimentally at least, a combination of skeletal resorption and vigorous bone-matrix formation (5–7). Also, in the hypoparathyroid individual, administration of vitamin D results in an increased excretion of phosphorus and usually of calcium (8, 9).

Knowledge of the biochemical or enzymatic means by which vitamin D induces the foregoing effects would greatly enhance our understanding of mineral and bone metabolism. An approach to the study of this problem was afforded by the reports of Warkany and his co-workers (10–12) on bioassays of vitamin D in sera of animals and humans. Vitamin D in aqueous solution, however, is known to be readily oxidized and thereby rendered inactive (13). This suggests that the vitamin, as it exists in serum, is in some manner protected and may not be in a free or unaltered state. Therefore, in our initial studies on vitamin D it seemed pertinent to first survey the activity of this vitamin in the sera of patients having various disorders of calcium metabolism, and then to investigate its mode of transport and some of its physical properties as it exists in serum.

METHODS

Technique of bioassay. The amount of vitamin D normally present in serum is so small (10–12) that inconveniently large volumes of blood are required when bioassays are conducted by the usually recommended methods (14). It was determined that such requirements could be reduced by *1*) designing a program of dosage inducing a maximum response from a minimum of serum, and *2*) by utilizing a ricketogenic [1] test diet which would result in uniformly severe rickets so as to lessen the number of test-failures requiring repetition. To meet these aims the following procedure was devised.

A colony of rats (Sprague-Dawley strain) was maintained in conformity with the general requirements of experimental rickets (14). The rats used for test purposes were weaned at a weight of 40 to 45 Gm. and were then fed the ricketogenic diet (Diet F, below) for 21 days. These rats were then put into individual cages and were given a special feeder containing the test substance. The feeder consisted of a tin pill box (50 mm. wide) cemented into a Petri dish, the space between the rim of the box and the rim of the dish serving as a moat which minimized the loss of scattered food. Three Gm. of Diet F and 0.2 ml. of maize oil were triturated in the pill box, and 2.0 ml. of serum, or serum diluted with water, was stirred in. Within an hour the test preparation formed a stiff gel which was difficult for the rat to scatter. Prompt consumption of the test substance was favored by presenting it in the late afternoon and it was usually eaten by the following morning. Rats not consuming all of the test material within 24 hours were discarded. Since the amount of food containing the serum for assay was less than half of the normal daily intake, such failures did not often occur. When reference solution of vita-

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[1] The etymology of this term has been discussed in footnote 1, Reference 13, and the recommended spelling has been adhered to in this article.

min D (U.S.P. Reference Standard) was employed, it was given in like manner, *i.e.*, in the 0.2 ml. of maize oil plus 2.0 ml. of water triturated with 3.0 Gm. of Diet F. Rats eating the assay mixture within the requisite 24 hours were then maintained on the ricketogenic Diet F until time of sacrifice.

It was found that good responses could be obtained in a total test period of five days (one day of vitamin D or serum supplementation followed by four days on plain Diet F *ad libitum*). All assays reported herein were done on this basis. The tibiae of the rats were examined for healing rickets after staining with silver in accordance with the usual line test technique and the degree of healing was assessed on a scale of five degrees as previously described (14). The curative action of the sera tested was evaluated by determining the amount of vitamin D required to induce the same healing effect. It has been our experience that with Diet F, employed as described, an assay of human serum can be performed with approximately half as much serum as would have been required in a conventional assay of comparable accuracy. The assay method utilized in this study permitted the detection of as little as one unit, or 0.025 μg., of vitamin D.

Ricketogenic Diet F. The diet used was of the following composition (Gm. per Kg. of diet): maize starch, 500; dextrose (hydrous), 200; fibrin[2] (extracted with hot alcohol), 150; dried yeast (Torula[3] or brewer's[4]), 40; precipitated calcium carbonate, 30; salt mixture,[5] 10; vitamin A palmitate in maize oil (100 I.U. per ml.), 70.

All ingredients were of sufficient fineness to pass through a 40-mesh sieve. The maize oil was added to the other components during slow, mechanical mixing and the diet, blended in 6 Kg. quantities, was stored under refrigeration.

Diet F is nutritionally complete except for a deficiency in phosphorus. The rickets induced in 21 days on this diet is much more severe and more uniform than that produced in the same period by use of conventional ricketogenic diets. The metaphyses are wide, free of calcified spots and rarely show cupping.

Serum. Serum not subjected to bioassay on the day of withdrawal was, except as noted below, kept in the frozen state until use.

Permeability studies. Ultrafiltration of serum was conducted according to a previously published technique (15) and both the ultrafiltrate and residue were assayed for antiricketic activity. Visking cellophane tubing was used to contain the serum during 48 hours dialysis against physiologic saline at 4° C.

Electrophoresis. Electrophoresis of serum was conducted with starch blocks ($10 \times 45 \times 0.5$ cm.) at 4° C. (16). Four ml. of serum containing a small amount of bromphenol blue and 1 ml. of barbital buffer was carefully placed near one end of the starch block. Under the conditions maintained (constant power source of 350 V. and 30 ma.) a 40 cm. migration of the serum was obtained in 38 to 40 hours. The starch block was then divided into 1 cm. strips which were placed in individual centrifuge tubes containing 3 ml. of cold 0.9 per cent NaCl. The contents of each tube were immediately mixed, centrifuged and the supernatant liquor was analyzed for concentration of protein (17). The remainder of the liquor was frozen for subsequent bioassay.

[2] General Biochemical, Inc., Chagrin Falls, Ohio.

[3] Rhinelander Paper Company, Rhinelander, Wisc.

[4] Anheuser-Busch, Inc., St. Louis, Mo.

[5] The salt mixture consisted of (Gm. per Kg. of mixture): iodized table salt, 500; potassium chloride, 440; ferric phosphate, 20; magnesium carbonate, 20; manganese sulfate (dried), 17; cupric sulfate (anhydrous), 3.

TABLE I

Antiricketic activity of serum from hypoparathyroid patients receiving vitamin D_2

Patient	Daily dose of D_2	Duration Rx	Serum Ca	Assay
	(*I.U.*)		(*mg. %*)	(*I.U./ml.*)
E.P.	50,000	1 yr.	9.5	40
A.M.	50,000	2 yrs.	8.4	40
L.L.	100,000	6 mos.	10.6	30
A.C.	100,000	5 yrs.	10.0	48
M.R.	100,000	2 yrs.	9.1	50
H.C.	200,000	15 mos.	9.6	18
L.C.	200,000	3 yrs.	8.6	8

RESULTS

Normal and hypoparathyroid subjects

The sera of 18 normal subjects were assayed and found to contain between 0.7 and 3.1 I.U. of antiricketic activity per ml. (mean of 2.0 I.U. per ml.). These results are essentially in agreement with those previously reported by Warkany and his associates (10–12). Hypoparathyroid patients receiving 50,000 to 200,000 I.U. of vitamin D_2 daily had much more antiricketic activity in their sera, from 8 to 50 units per ml. (Table I). However, as is evident from the results recorded in Table I, the amount of antiricketic activity existing in the sera of these patients could not be accurately predicted from either the dose of vitamin D or the concentration of calcium in the serum.

It is known that persons given large doses of vitamin D have, after cessation of dosage, a slow decline in their serum activity (10–12). Observations on two of our subjects illustrate the variation which may occur in the persistence of serum antiricketic potency. One D-intoxicated patient with 30 units per ml. in her serum still had an activity equivalent to 6 units per ml.

seven months after D administration was stopped. Another patient intoxicated by vitamin D and with a serum calcium concentration of 15 mg. per 100 ml. had an activity of only 8.5 units of vitamin D per ml. of serum. In this latter patient the serum concentration was normal (2 I.U. per ml.) two and a half months after cessation of vitamin therapy. The slow decrease in antiricketic activity of serum subsequent to prolonged dosage with vitamin D is to be compared with the rate of increase in serum activity on *institution* of the vitamin. When to a normal subject 100,000 I.U. of D_2 was given orally per day, the increase in serum activity was detectable within eight hours (activity increased by 2 I.U. per ml.), and at one week with continued dosage the serum D concentration was found to be equivalent to 14 units per ml. These and the previously cited observations are in keeping with clinical evidence that vitamin D is a cumulative drug (9).

Hypercalcemic subjects

Sera from patients having hypercalcemia in association with a variety of disorders were subjected to bioassay. As was to be expected the sera from three patients with hyperparathyroidism had a normal vitamin D content. Similarly, the antiricketic activity of sera from eight patients having hypercalcemia in association with various neoplastic diseases (carcinoma of the lung, multiple myeloma and undifferentiated carcinoma of the pelvis) was also normal. Likewise, serum obtained from a single patient hypercalcemic with the milk-alkali syndrome did not contain an increased amount of vitamin D.

TABLE II

Antiricketic activity of serum in nontropical sprue

Patient	Therapy	Date	Serum Ca (mg. %)	Serum P (mg. %)	Assay (I.U./ml.)
M.S.	None	2/6/54	5.2	1.3	1.1
	Cortisone & Ca	5/24/56	9.3	5.0	0.5
M.T.	None	2/5/57	4.5	2.4	0
	Cortisone (1 wk.) & Ca	2/19/57	6.9	1.6	0.7
	Cortisone & 150,000 I.U. D_2/day for 3 mos.	5/21/57	8.0	3.6	1.3
	Cortisone & 150,000 I.U. D_2/day for 7 mos.	9/10/57	5.7	3.9	1.3
M.Q.	Cortisone & 100,000 I.U. D_2 for 2 yrs.	3/15/56	9.8	2.9	31
	No D_2 for 6 wks.	8/10/56	8.6	3.7	7

Idiopathic hypercalcemia of infants has many clinical similarities to D intoxication and is apparently not an uncommon disorder in England where milk is more heavily fortified with vitamin D than it is in the United States. This serious malady has been suspected to be due to vitamin D intoxication (resulting from excessive intake or possibly increased sensitivity) (18–20), and it has been recently reported that three infants with this disorder had much increased concentrations of antiricketic activity in their sera (21). However, the lyophilized samples of sera (unaltered sera was not available) from two such hypercalcemic infants tested by us contained normal quantities of antiricketic substance (*vide infra*). Similar assay results were obtained with the sera from three patients having hypercalcemia in association with sarcoidosis, a disorder in which the suggestion of increased sensitivity to vitamin D has also been made (22, 23).

It is known that cortisone promptly reduces the hypercalcemia of *most* D-intoxicated persons and also that due to a number of other causes (24–26) (except, in our experience, hyperparathyroidism and lung carcinoma). In two hypoparathyroid patients made hypercalcemic with vitamin D, cortisone administration restored the serum calcium to normal, but *not to the original hypocalcemic concentrations.* Despite this decrease in calcium concentration the D content of the sera from these two patients remained unchanged by the cortisone. Such observations do not elucidate the means by which cortisone lessens hypercalcemia, but they are consistent with experimental studies indicating that vitamin D and cortisone have independent effects on bone (27–29).

Patients with nontropical sprue

Evidence exists of defective absorption of fat soluble vitamins in patients with nontropical sprue (30). Moreover, hypocalcemia is not infrequently present (31). In Table II are recorded the data on three patients with this disorder. Patient M.S. had marked hypocalcemia despite a normal amount of vitamin D in her serum. Patient M.T. had a barely detectable amount of D in her serum prior to vitamin administration, with only a slight increase in serum antiricketic activity after pro-

longed dosage with large amounts of vitamin D. Moreover, even though attaining a normal amount of D activity in her serum, she remained hypocalcemic. The third patient (M.Q.) developed mild hypocalcemia (with normal serum proteins) in the face of a rather high concentration of antiricketic activity persisting in her serum after cessation of D therapy. Thus, factors other than vitamin D deficiency must have been responsible for the hypocalcemia in these patients.

Physical properties of antiricketic substance in serum

Sera from a number of subjects were assayed immediately after separation from blood and again after being in the frozen state for various periods. The results of these studies indicated that there was no loss of activity of sera kept frozen for intervals up to one year. Moreover, additional observations demonstrated that maintenance of sera at room temperature for as long as three days prior to assay did not result in any loss of antiricketic potency.

Ultrafiltrates of three sera were found to be free of antiricketic activity whereas the nonultrafiltrable portions contained all the activity present in the original sera. Assays of the residue and ultrafiltrate of one sample of serum to which vitamin D_3 (in propylene glycol) had been added *in vitro* (500 I.U. to 20 ml. serum) gave similar results in that here, too, all of the vitamin D activity remained in the nonfiltrable fraction. Studies on serum from another patient receiving large amounts of vitamin D_2 revealed that the antiricketic substance was also nondialyzable.

Warkany (10) has reported that lyophilized serum maintained at $-20°$ C. retained all of its antiricketic potency. Using similarly treated sera which were reconstituted immediately prior to assay, we were able to confirm these observations. However, other studies which we have not yet completed suggest that in such reconstituted sera the antiricketic substance is not nearly so stable as in original sera. It is possible that loss of activity from lyophilized sera kept at room temperature accounted for the normal values obtained by us in hypercalcemic infants whereas others (21) have reported increased activity in such infants.

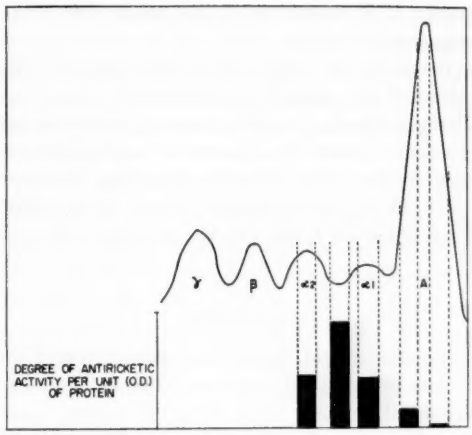

FIG. 1. CORRELATION OF ANTIRICKETIC ACTIVITY WITH AMOUNT OF PROTEIN PRESENT IN INDIVIDUAL FRACTIONS OF SERUM

Localization of antiricketic substance in serum

Sera from seven patients treated with large doses of calciferol were subjected to starch block electrophoresis. When eluates of the starch strips were fed to test rats, the antiricketic activity was located predominantly in the area of the alpha globulins and to a lesser degree in the albumin fraction; none was found to be with the slower moving globulins. Quantitation of the antiricketic effect (Figure 1) indicated that the greatest activity per unit of protein was in the area of the nadir between alpha 1 and alpha 2 globulin; next, in the alpha 1 and alpha 2 globulin-containing fractions; and least, in the albumin fraction.

To explore further the mode of transport of vitamin D, normal serum to which 500 I.U. of vitamin D_3 in propylene glycol had been added *in vitro* was also subjected to starch block electrophoresis. The location of the antiricketic activity was the same in this serum as in the sera from patients given the vitamin orally. In addition, we were unable to locate any antiricketic activity in starch strips after attempted electrophoresis of vitamin D_3 in propylene glycol alone.[6] Therefore, it is probable that the electrophoretic mobility of

[6] Whether this lack of activity was a result of migration of D_3 off the starch block or of inactivation is not known.

vitamin D is dependent on its union with some component of serum.

One series of observations was made on sera fractioned by alcohol precipitation.[7] Assay of 250 mg. aliquots of such fractions of pooled human sera demonstrated the presence of antiricketic activity in the alpha globulin-containing fractions (IV-1, IV-4) and a minimal degree in the albumin fraction (V), but not in Fractions I, II and III.

DISCUSSION

Recently several studies have been reported on the fate of orally administered vitamin D in experimental animals (33, 34). Following such administration only a small fraction (10 to 30 per cent) of the dose could be recovered, the major portion having been destroyed, presumably within the intestinal lumen. It has been suggested that oxidation of the D vitamins within the gut may account for their disappearance after oral dosage and that the bacterial flora of the gut may be the responsible agents (33, 34). In one test of this possibility succinylsulfathiazole was fed to a group of rats prior to D administration, but this did not result in any improved recovery of the vitamin (35).

The results obtained on human subjects in the present experiments and previously, also, by Warkany (10–12) are consistent with the findings of Cruickshank and his co-workers (33–35). We found that ingestion of large doses of vitamin D were required to increase appreciably the antiricketic activity of the serum. Indeed, in the patients with hypoparathyroidism treated with vitamin D the lowest potency was found in the sera of those receiving the largest amount of vitamin (H.C. and L.C. of Table I). We have no data as to whether or not the *relatively* low serum activity in these particular patients resulted from poor absorption or from excessive destruction of the vitamin. Relevant to the possibility of increased destruction is the report of several patients given oral doses of 20 mg. (800,000 I.U.) of vitamin D_2 in whom almost no active vitamin was recovered from the feces on biological assay (36).

[7] Serum fractions prepared by alcohol precipitation (32) and kindly supplied by Dr. Kenneth L. Zierler.

All patients who became hypercalcemic on large doses of vitamin D had much increased concentrations of antiricketic activity in their sera, but in some of the treated patients even higher concentrations did not result in hypercalcemia. Increases in serum calcium concentration induced by vitamin D are known to be enhanced by immobilization of the patient and also by the ingestion of alkali or large amounts of calcium, but no such causes were recognized to account for the gradations in responsiveness of the above subjects.

There are a number of circumstances associated with hypercalcemia other than hypervitaminosis D and the normal values for vitamin D-like activity found in the sera of patients with some of these hypercalcemic states are not surprising. However, it should be emphasized that these sera did not contain abnormal quantities of antiricketic substance *detectable with the rat*. The method of assay used in these experiments consisted of a comparison of the antiricketic effect of test substance with the effects of known amounts of vitamin D in ricketic rats. One must recall that dihydrotachysterol has, *in man*, hypercalcemic effects approximately equal to those of calciferol mg. for mg. (37, 38), yet this compound is only 1/400 (or less) as potent an antiricketic substance as calciferol when fed to the rat (39, 40). Thus, our data do not negate the possibility that some agents differing only slightly from vitamin D may at times be etiologically important in the genesis of hypercalcemia.

The data obtained on the three patients with nontropical sprue were of interest and suggest that even though absorption of the vitamin may be poor such a defect alone is not sufficient to explain the hypocalcemia in these patients. Calcium absorption is known to be impaired in patients with steatorrhea and this may be of such degree and duration as eventually to result in hypocalcemia (apparently occurring when the bones become unable to support adequately serum calcium concentration). The reduced calcium concentration in the sera of patients given an exchange resin for weeks or months may be an analogous situation (41, 42).

Presumptive evidence that the vitamin D-like factor of serum is intimately bound to certain

macro-molecules is afforded by its stability in serum, its nonultrafiltrability and the characteristic electrophoretic mobility. Moreover, cellular activity is not required for the union of vitamin D with its carrier in serum. The specific components of serum, whether proteins, lipoproteins or other compounds bearing this antiricketic factor have not been determined, but it is noteworthy that most of the activity was located in an area which does not correspond to that of the *peak* concentrations of either alpha 1 or alpha 2 lipoproteins (43).[8]

Little is known of the biochemical processes whereby vitamin D affects calcium absorption or bone metabolism. The vitamin has been reported to inhibit anaerobic glycolysis (45) and to enhance cocarboxylase formation (46). Also, it apparently retards oxidation of citrate to α-ketoglutarate in the kidney but not in the liver (47, 48). These isolated biochemical observations do not distinguish direct from indirect effects of vitamin D and much more information is necessary to delineate the specific function of the vitamin as a coenzyme or necessary permissive agent in basic metabolic processes.

The observations reported herein on the antiricketic activity in serum, in addition to providing some insight into the variable response encountered on treating patients with vitamin D, has been helpful in the investigation of a number of disorders of calcium metabolism. Moreover, demonstration that the vitamin is transported with specific fractions of the serum proteins in a stable and not readily oxidizable form may serve as a basis for future study of the chemical nature of the carrier substance and the state of vitamin D in serum.

SUMMARY

The mean antiricketic activity of sera from 18 normal subjects was found to be equivalent to 2 I.U. of vitamin D per ml. Hypoparathyroid patients receiving vitamin D_2 had much increased activity in their sera, but the degree of activity could not be accurately predicted from either the dose of the vitamin or the induced increase in calcium concentration of the sera.

No increase in antiricketic effect was found in the sera of patients with various nonvitamin D-induced hypercalcemic states. Moreover, failure of vitamin D absorption alone did not account for the hypocalcemia in three patients with nontropical sprue.

The vitamin D activity of sera was found to be stable even at room temperature and was also nonultrafiltrable. On starch block electrophoresis the predominant antiricketic effect was limited to the area of the alpha globulins. The physical and electrophoretic characteristics were identical whether vitamin D was added to serum *in vitro* or *in vivo*.

ADDENDUM

Since submission of this manuscript additional studies have been conducted on the stability of reconstituted lyophilized sera. Assays of such sera revealed no loss of antiricketic potency after standing at room temperature for as long as three days. These observations suggest that the results obtained with lyophilized sera from hypercalcemic infants in Scotland are valid. Therefore, the assay data available, although limited, indicates that the genesis of the hypercalcemia in the Scottish children tested differs from that in the American infants reported by Fellers and Schwartz (21).

[8] It should be noted that others (44), using a method difficult to assess, have reported binding of vitamin D_2 to protein fractions different from those suggested by us.

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THE MECHANISM OF CLOT DISSOLUTION BY PLASMIN *

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Normal human and animal sera contain a globulin, plasminogen, which in the presence of activators is rapidly converted to plasmin, a proteolytic enzyme active at neutral hydrogen ion concentrations. Plasmin is an enzyme of wide specificity and will attack such varied substrates as gelatin, casein, certain synthetic esters, accelerator globulin, complement, fibrinogen and most importantly fibrin. Plasminogen activation occurs spontaneously (1), or as a result of contact with activators of tissue (2), body fluid (3, 4), or bacterial origin (5). The conversion of plasminogen to plasmin involves the loss of a peptide moiety and there is evidence to suggest that the plasmin obtained by different modes of activation may vary in composition (6).

Biochemically, considerable species differences exist not only between the plasminogen system of man and animals, but more particularly between the systems of various animals; this variability is most extreme with regard to the differential effectiveness of streptokinase. Rigid kinetic studies (6) reveal that the activation of human plasminogen under the influence of streptokinase (an extracellular product of hemolytic streptococcal metabolism), trypsin and urokinase (prepared from human urine) results from a first order enzymatic reaction.

Since physiological fibrinolytic phenomena result from activity of the plasminogen system, attempts to use plasmin or plasminogen activators to effect therapeutic thrombolysis have been numerous. Animal experiment, despite the difficulties and confusion of species variability, has yielded striking findings (7, 8, 9). Though the demonstration of experimental thrombolytic action has been of an unequivocal nature, the precise mechanism of its production has hitherto been obscure.

The present communication describes *in vitro* experiments and *in vivo* observations bearing upon thrombolytic [1] mechanisms in man. The results indicate that since plasminogen is found both in plasma and also as a constituent of thrombi, clot lysis occurs by a dual mechanism. The chief and primary mechanism of thrombolysis involves the diffusion or adsorption of plasminogen activator to the thrombus, activation of intrinsic clot plasminogen and thrombolysis. The secondary mechanism involving digestion of the thrombus by extrinsic plasmin action appears to be of little quantitative importance.

MATERIALS AND METHODS

Streptokinase [2] was a highly purified preparation, biophysically though not immunochemically homogenous; the specific activity was 600 to 700 streptokinase units per μg. nitrogen.

Human plasminogen (1) contained 100 to 150 casein units per mg. tyrosine.

Plasmin was prepared by autocatalytic activation of plasminogen in glycerol (1) and contained 95 casein units per mg. tyrosine (see Casein assay).

Epsilon amino caproic acid [2] (6-amino hexanoic acid) had a melting point of 208° C. and an elemental analysis corresponding in composition to that of the pure compound.

Urokinase [3] contained 5,100 Ploug units per mg. dry weight (10).

Fibrin plate test was modified from Müllertz (11) by using a highly purified fibrinogen preparation [4] in a final concentration of 0.2 per cent. *Heated fibrin plates* (12)

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[1] The term thrombolytic is used to designate a process by which preformed clots are lysed. This usage corresponds to the literal meaning and original derivation of the word (Oxford English Dictionary) and its use does not imply specific reference to pathological states where intravascular thrombi may be of variable composition.

[2] Kindly supplied by Dr. J. Ruegsegger, Lederle Laboratories, Pearl River, N. Y. Streptokinase activity expressed in Christensen units (J. clin. Invest. 1949, **28**, 163).

[3] Kindly supplied by Dr. J. Ploug, Leo Pharmaceuticals, Copenhagen, Denmark.

[4] Bovine fibrinogen, salt free, 92 per cent of the nitrogen being clottable by thrombin; kindly supplied by Dr. Kent Miller, New York State Institute of Health, Albany, N. Y.

were prepared by subjecting fibrin plates to 80° C. for 30 minutes. This procedure resulted in denaturation of the contaminating plasminogen, rendered the plates insusceptible to the action of plasminogen activators, but also reduced their sensitivity to proteolysis (pure chymotrypsin [5] 0.05 mg. per ml. gave a zone of 473 mm.^{2} on unheated plates and 276 mm.^{2} on heated ones, while the figures for spontaneously activated plasmin (1.3 casein units per ml.) were 350 mm.^{2} and 172 mm.^{2}, respectively).

Fibrinogen was estimated by the method of Ratnoff and Menzie (13) modified only by the addition of 0.1 ml. of 1 per cent soy bean trypsin inhibitor [5] to prevent further fibrinolysis.

I^{131} labeled plasma clot test. Fibrinogen [4] was iodinated by a method modified from that of Eisen and Keston (14). Assuming a molecular weight for fibrinogen of 350,000 (15), the degree of iodination varied between 0.6 and 1.3 I^{131} atoms for each molecule of fibrinogen. Free I^{131} was removed by passage through an Amberlite IRA-400 column (40 mesh) and integrity of the fibrinogen molecule demonstrated by the fact that after iodination treatment the nitrogen clottable by thrombin was 92 per cent (the starting value). Eighty-nine to 93 per cent of the radioactivity was clottable by thrombin.

Preformed isotopically labeled human clots were made by adding 1 to 4 λ of the iodinated fibrinogen (1 to 2 per cent solution) to 0.5 ml. of bulk plasma containing added plasminogen (*vide infra*) in an 8×80 mm. serological tube. The quantity of added radioactivity was calculated to give approximately 100 cpm per μg. of contained fibrinogen. For convenience, throughout the test, observed counts per minute have been mathematically transformed so that a single count per minute corresponds to the lysis of 0.8 μg. of fibrin. A wire, nichrome 26 S. W. G., coiled at its lower end, was inserted into each tube and 0.1 ml. of thrombin (50 units per ml.) was added. The clots were incubated for one to two hours in a 37° C. water bath and after retraction were withdrawn by means of the wire. Approximately 50 such clots were washed in 2 L. of normal saline overnight at 2° C. This procedure resulted in a low and consistent blank value for release of radioactivity.

I^{131} labeled plasminogen deficient, plasminogen normal and plasminogen rich clots

Plasminogen deficient clots. I^{131} labeled human plasma clots vigorously washed in a large excess of phosphate saline buffer (0.1 M) at pH 7.6 for 24 hours at 2° C. were shown to contain less than 0.15 casein units of plasminogen per clot (unwashed drained clots contained 0.3 to 1 casein unit). These clots were relatively resistant to lysis by plasminogen activators, while retaining a normal sensitivity to the action of plasmin. Residual plasminogen could be denatured by heating these clots in buffer at 80° C. for 30 minutes. Such clots, designated as "heated clots," were completely resistant to lysis by plasminogen activators but the heating process had reduced their sensitivity to the action of plasmin so that it was approximately 60 per cent of that shown by unheated clots.

Clots of normal and high plasminogen content. Since clots had to be washed to reduce blank radioactivity values and this process reduced clot plasminogen, the preparation of clots of normal plasminogen content required reinforcement of the original plasma with purified plasminogen. This problem was simplified by the fact that while plasma plasminogen could be washed from plasma clots, purified plasminogen, owing to its different physical state of dispersion at pH 7.6, was completely taken up from the plasma into the clot and was not washed out. Plasma clots of normal plasminogen content were made by fortifying the original plasma with 0.5 to 1 casein unit plasminogen per clot. Plasminogen rich clots were made similarly except that the added plasminogen was 2 casein units plasminogen per clot. These latter clots were extremely sensitive to the presence of plasminogen activators and exhibited normal sensitivity to lysis by plasmin.

Thrombolytic [6] *assays* were made by inserting a prepared clot into 0.5 ml. of plasma or other specimen in a 10×100 mm. tube and incubating at 37° C. for 30 minutes. The clot was then withdrawn, removed from the wire and centrifuged in 2 ml. of normal saline; the supernatant was added to the residual plasma and the radioactivity determined in a well-type scintillation detector (efficiency 45 per cent). Comparison of residual clot nitrogen content with residual radioactivity demonstrated that clot lysis and radioactivity release were highly correlated.

Assay of isotopically labeled clots for plasminogen. Single clots were ground with powdered glass in a tube containing 0.5 ml. 1/6 N hydrochloric acid. After 15 minutes at room temperature, the supernatant solution was neutralized and assayed for plasminogen by the casein method. The validity of this assay procedure was tested on a series of 15 plasmas, to some of which had been added plasminogen and to others fibrinogen. The mean recovery of plasminogen from clot plus supernatant serum was 94 ± 9 per cent (range 79 to 109 per cent) of the original plasma aliquot.

Assay for plasminogen and plasmin in plasma

Casein assay. The casein assay (16) was modified from that of Remmert and Cohen (17), our unit being

[5] Worthington Biochemical Corp., Freehold, N. J.

[6] The presence of plasma inhibitors requires that a clear distinction be drawn between total demonstrable activator or enzyme activity and that portion that is physiologically active (uncombined with inhibitor). Test systems requiring dilution or other changes in biophysical conditions are unacceptable for the assay of physiologically active components in the complex milieu of plasma, since these changes alter the ratios of combined to uncombined activities. Assay of these individual activities in undiluted plasma by means of isotopically labeled plasma clots of varying plasminogen concentration fulfills these stringent requirements.

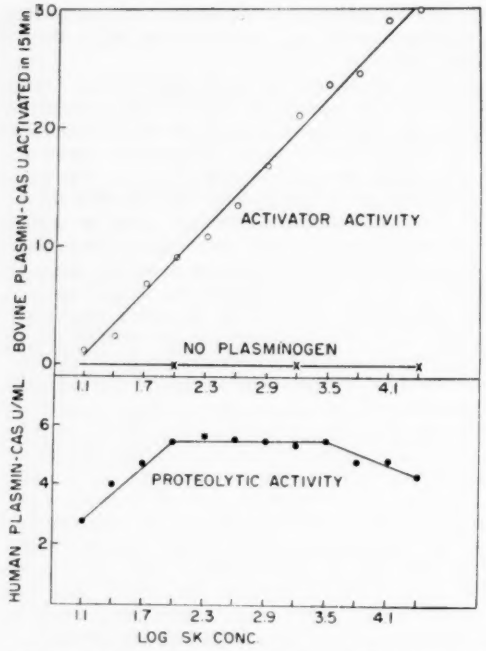


FIG. 1. ACTIVATOR AND PROTEOLYTIC ACTIVITIES OF HUMAN PLASMINOGEN AT VARIOUS STREPTOKINASE (SK) CONCENTRATIONS

Plasminogen concentration, 5.6 casein units per ml. Activator activity was approximately related to the logarithm of the streptokinase concentration (indicated on the figure as streptokinase log units per ml.) and was independent of the proteolytic activity.

aproximately 50 per cent that of the latter authors. Assay of untreated plasma for plasminogen yielded low values and recovery of added plasminogen was incomplete owing to the presence of antiplasmin activity in untreated plasma. However, direct assay of plasma antiplasmin content revealed that this activity was largely or completely destroyed by adding 0.5 ml. of 1/6 N hydrochloric acid to 0.5 ml. of plasma, leaving at room temperature for 15 minutes and then neutralizing with 0.5 ml. of 1/6 N sodium hydroxide and 1 ml. of 0.1 M phosphate buffer at pH 7.6. With this modification recovery of added plasminogen or plasmin became quantitative and assay values for plasma plasminogen were substantially increased. All plasma used for casein assay was treated in this manner.[7]

[7] The use of this method for the assay of plasmin in plasma may, under certain conditions, yield spuriously high values. There is evidence that, if the plasma contains both activator and plasminogen, the plasma manipulation (dilution, and so forth) consequent upon the assay procedure may facilitate plasminogen activation.

Esterase activity was determined by the hydrolysis of benzoyl arginine methyl ester (BAMe) (18). The degree of hydrolysis was measured by the Hestrin colorimetric method (19). In contrast to the findings with casein plasminogen assay, prior acidification of the plasma was unnecessary if BAMe were used as a substrate. Though the reason for this discrepancy is uncertain,[8] the experimental difference has been adequately validated.

Plasminogen "activator" assay. The activation of plasminogen by streptokinase occurs as a two stage reaction (20, 21). Streptokinase first reacts with a plasma protein, termed proactivator,[9] to form activator and the activator converts plasminogen to plasmin by enzymatic transformation (20, 21). Since proactivator is absent from bovine plasma, bovine plasminogen may be used to assay "streptokinase activator" present in the plasma of other species.

Five-tenths ml. of bovine Fraction III_2 (Armour) (20 mg. per ml. or 5.6 casein units per ml.) was incubated for 15 minutes at room temperature with 0.1 ml. of the test solution. The activation reaction was then stopped by the addition of epsilon amino caproic acid to a final concentration of 0.08 M (16) and the solution assayed for plasmin activity by the casein assay. The results were expressed as casein units bovine plasminogen activated per 15 minutes.

RESULTS

The action of streptokinase on plasminogen preparations

Figure 1 illustrates activator and proteolytic activity assays made after the addition, 15 minutes previously, of increasing quantities of streptokinase to a purified human plasminogen preparation. It is evident that the two distinct activities of plasminogen preparations developed under the influence of streptokinase, the proteolytic and the activator activities, are differently related to the streptokinase concentrations employed. Whereas, proteolytic activity reaches a plateau at 100 streptokinase units per ml., which is maintained with

[8] Presumably the affinity of plasmin for BAMe is greater than for antiplasmin.

[9] There is still controversy as to whether proactivator is an entirely separate substance or is derived from plasminogen itself (22, 23, 24), but the term is used to designate a plasma factor capable of reacting with streptokinase to form plasminogen activator. Though divergent emphasis has been placed on the significance of proactivator in the human as apart from the bovine system (25, 26, 27) for descriptive purposes we acknowledge its separate existence and assay for its biochemical activity even though its individual identity apart from plasminogen or its derivatives has not been clearly established.

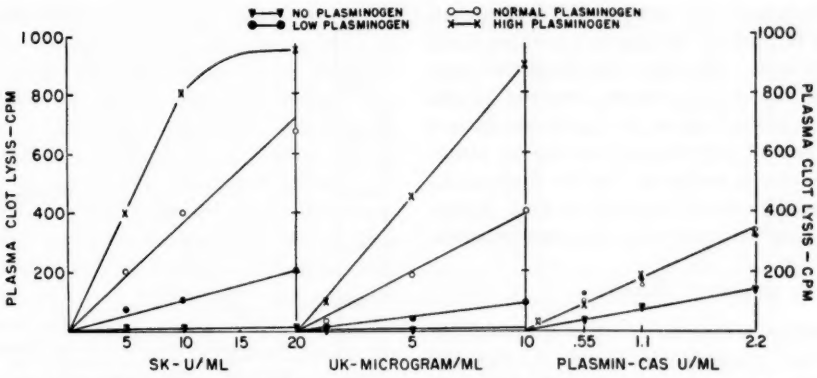


FIG. 2. THE LYSIS RATE OF I^{131} TAGGED PLASMA CLOTS AS A FUNCTION OF THE PLASMINOGEN CONTENT OF THE CLOT AND OF THE ACTIVATOR AND PLASMIN CONCENTRATIONS

Lysis rates for streptokinase and urokinase varied as the logarithm of plasminogen concentration being zero in clots devoid of plasminogen. Lysis rates with plasmin were uninfluenced by the clot plasminogen concentration except that where clots were devoid of plasminogen, a lesser rate of lysis was found, due to denaturation caused by the heating process.

increasing streptokinase concentrations until it ultimately commences to decline around 3,000 streptokinase units per ml., activator activity behaves differently. Activator activity continues to increase with increasing streptokinase concentrations and is still rising at the highest streptokinase concentration used (25,000 streptokinase units per ml.). Since thrombi normally contain significant quantities of plasminogen, it is apparent that under the influence of streptokinase, plasminogen preparations develop two types of activities, proteolytic and activator, either of which is potentially capable of mediating the lysis of a thrombus.

The effect of streptokinase, urokinase and plasmin upon plasma clots

Figure 2 shows the rate of supernatant radioactivity release determined when isotopically labeled plasma clots of differing plasminogen concentration (four concentrations displayed in each panel) were incubated at 37° C. for 30 minutes with several concentrations of streptokinase, urokinase and spontaneously activated human plasmin (in 0.1 M phosphate buffer at pH 7.6). The assay technique was that described under the heading of thrombolytic assay in the Methods section.

The results for streptokinase and urokinase (Panels 1 and 2) show that not only is the rate of clot lysis linearly related to the surrounding activator concentration,[10] but that it is also, at any given activator concentration, related, though not directly proportional, to the clot plasminogen concentration. In fact, in this and other experiments, over a "physiological" range of clot plasminogen concentrations, clot lysis rates at a given activator concentration have been approximately proportional to the logarithm of the clot plasminogen concentration. It is evident that clots lysing in a streptokinase solution do so because there are ample amounts of proactivator as well as plasminogen in the clot.

Panel 3 illustrates the effect of spontaneously activated human plasmin. The lysis rate rises linearly with enzyme concentration but the release of radioactivity was independent of clot plasminogen concentration. Though clots of high, normal and low plasminogen concentration were lysed at identical rates, the lysis rates of those clots marked "no plasminogen" were about half those of the others. This occurred as a consequence of their method of preparation, which involved heating, and the diminished sensitivity to plasmin action was similar to that recorded with the heated fibrin plates (see Methods section). The sensitivity of the unheated clots to the action of plasmin in

[10] This relationship should not be interpreted as favoring either of the two alternative views concerning passive diffusion of or active absorption of streptokinase or urokinase to a clot.

terms of fibrin lysis rates was one-fifth to one-tenth that shown by purified fibrinogen or purified fibrin in a diffuse state. Whether this diminished sensitivity was due to the relative impurity of the fibrin in the washed clot or due to the mechanical barrier to enzyme penetration posed by the structure of the clot is uncertain, but the findings do suggest that digestion of thrombi, *in vivo*, by extrinsic plasmin action may be somewhat impeded.

Thrombolytic activity in plasma

The results of adding an excess of streptokinase to plasma are illustrated in Figure 3. Seventy streptokinase units were added per ml. of plasma, but since the antibody content of the plasma was equivalent to 30 streptokinase units per ml., the results illustrated refer to the presence of 40 free streptokinase units per ml. plasma. The upper portion of the figure shows that rapid activation

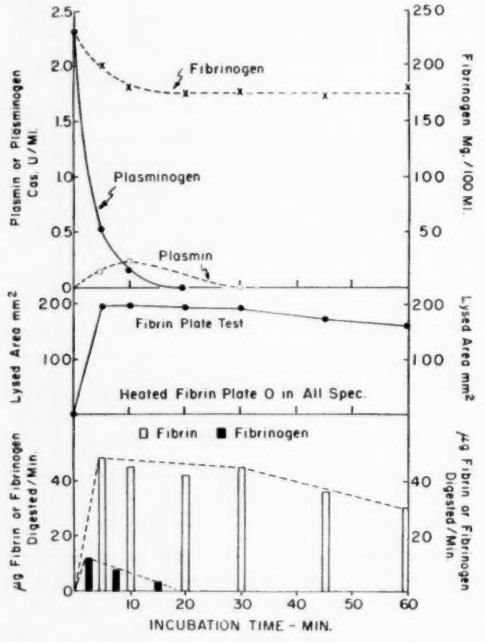


FIG. 3. BIOCHEMICAL CHANGES PRODUCED BY STREPTOKINASE IN PLASMA

Seventy streptokinase units were added per ml. plasma at zero time. The plasma activity developed was primarily thrombolytic in nature and fibrinogenolysis was minimal. This comparison is most strikingly seen in the bottom section of the figure.

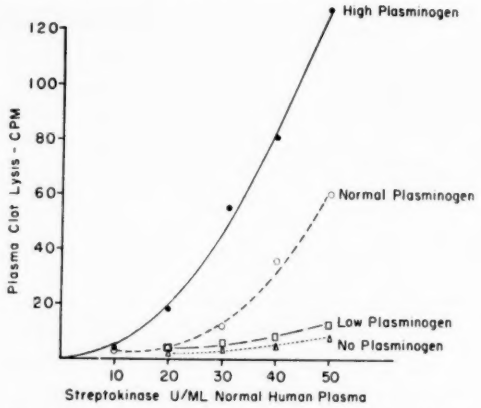


FIG. 4. THE DEPENDENCE OF STREPTOKINASE EFFECT UPON CLOT PLASMINOGEN CONCENTRATION

Clots devoid of plasminogen were unaffected by streptokinase treated plasma. Clots containing plasminogen were lysed by streptokinase treated plasma at rates dependent upon their plasminogen concentration.

of plasminogen occurred, so that at the end of 10 minutes only 10 per cent of the original quantity remained and after 20 minutes a zero assay reading was obtained. Concomitantly with the fall of plasminogen concentration, plasmin activity of low degree was detected, but it is noteworthy that this activity was only detectable after plasma antiplasmin activity had been destroyed by acidification (see Methods section). Assay of unacidified plasma for plasmin activity (not illustrated in Figure 2) revealed the presence of little or no proteolytic activity.

Fibrinogen levels declined to 75 per cent of their original value and then became stable; these assay values have been calculated as rates of fibrinogen lysis (μg. lysed per minute) and are plotted as such in the lower portion of Figure 3. However, in contrast to the low degree and short duration of plasma fibrinogenolytic activity, assay for plasma thrombolytic activity by means of the isotopically labeled clot technique (using clots of normal plasminogen content) disclosed an intense and sustained state of clot lysing activity. This activity, calculated as rates of fibrin lysis (μg. clot fibrin lysed per minute), has been plotted in the lower portion of Figure 3, where it may be compared with the rate of fibrinogen lysis calculated similarly. Comparison of these biochemical activities shows extreme divergence

between their magnitudes and durations, plasma clot lysing power being predominant in both respects. The fibrin plate assay results, displayed in the middle portion of Figure 3, confirm this conclusion. Assay on unheated fibrin plates showed a high and sustained degree of plasma thrombolytic activity that paralleled that revealed by the isotopic clot dissolution test. However, all readings made on heated fibrin plates were zero, indicating a plasmin activity of less than 0.15 casein unit per ml. (sensitivity limit of the method).

The biochemical findings illustrated in Figure 3 are typical of those found on addition of streptokinase to plasma. Variation of the streptokinase concentration, at least within the range of five to 50 free streptokinase units per ml. plasma, while altering the speed and degree of the reaction does not influence its general qualitative nature.

The relationship of thrombolysis, plasma thrombolytic activity and clot plasminogen concentration

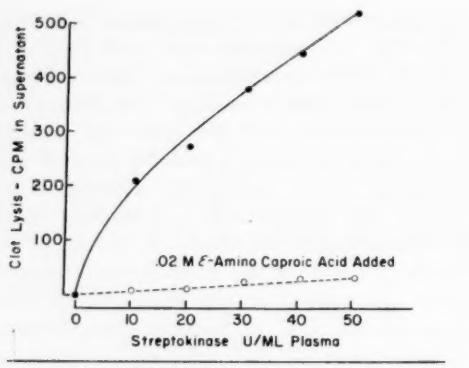
Figure 4 shows the degree of lysis produced in clots of differing plasminogen content immersed in plasma containing varying concentrations of streptokinase. The four clot plasminogen concentrations tested were those used in the experiment shown in Figure 2 and the plasma antibody content was equivalent to 15 streptokinase units per ml. Lysis failed to occur in any of the clots immersed in plasma containing 10 streptokinase units per ml. and it has been an invariable finding that significant thrombolysis has always been absent unless the quantity of added streptokinase has been sufficient to exceed the antibody binding power of the plasma. Though under special circumstances using different techniques a limited dissociation of the streptokinase antibody complex can be demonstrated, provided that the system is not diluted, dissociation of the streptokinase antibody complex exerts no practical effect.

The assays conducted at streptokinase concentrations of 20, 30, 40 and 50 units per ml. plasma showed an increasing divergence between the lysis rates of clots containing different plasminogen concentrations. Where clot plasminogen was low or absent, thrombolysis was negligible or absent. In contrast, clots containing a normal plasminogen concentration were rapidly lysed and this action was greatly enhanced if plasminogen rich clots were used. Thrombolysis rates with clots of a given plasminogen concentration were approximately linear with increasing concentrations of free streptokinase and the somewhat concave curves shown in Figure 4 probably resulted as a consequence of the varying time relations of reactions in the complex matrix of plasma. As in the earlier experiment shown in Figure 2, the rates of clot lysis, at any given activator concentration, were approximately related to the logarithm of the clot plasminogen concentration, though the data fitted this relationship far less exactly than in the former case.

These experimental findings, which closely mimic those found *in vivo*, demonstrate that clot lysis, at a given activator concentration, is dependent upon the plasminogen content of the clot. A corollary to these findings indicates that thrombolysis, at least under physiological conditions, must be dependent upon activation of intrinsic clot plasminogen. Moreover, the illustration that plasminogen deficient clots are lysed poorly, if at all, by streptokinase activated plasma speaks strongly against the concept that thrombolysis is related to extrinsic plasmin action under physiological circumstances.

The influence of an inhibitor of plasminogen activation upon thrombolytic activity displayed by streptokinase activated plasma

Plasminogen activation, in the presence of plasminogen activators, is inhibited competitively by low concentrations of epsilon amino caproic acid, themselves insufficient to inhibit plasmin action (16). Figure 5 (lower portion) displays such evidence, obtained by the fibrin plate method and pertaining to the experimental conditions used in the upper portion of Figure 5. The lower part of the figure shows that the high assay reading produced by plasma containing 50 streptokinase units per ml. was completely abolished by the addition to the mixture, prior to assay, of 0.02 M epsilon amino caproic acid. The action of plasmin on the plate, adjusted in amount so as to yield a zone of lysis comparable with that of the streptokinase activated plasma, was virtually unaffected by the addition of 0.02 M epsilon amino caproic acid.



Astrup Fibrin Plate Test:

Plasma + 50 SK U/ML	325 mm²
Plasma + 50 SK U/M + .02 M ε-Am Cap A.	0
Plasmin	390 mm²
Plasmin + .02 M ε-Amino Caproic Acid	360 mm²

FIG. 5. EFFECT OF ε-AMINO CAPROIC ACID ON PLASMINOGEN ACTIVATOR (MEASURED ON PLASMINOGEN RICH PLASMA CLOTS)

The bottom portion of the figure demonstrates that ε-amino caproic acid added to streptokinase treated plasma in a concentration of 0.02 M acts solely to inhibit plasminogen activation. The top portion of the figure demonstrates the abolition of thrombolytic activity in streptokinase treated plasma to which ε-amino caproic acid has been added.

The upper portion of Figure 5 shows that the powerful thrombolytic action of plasma containing varying concentrations of streptokinase on plasminogen enriched clots was totally suppressed by the addition of 0.02 M epsilon amino caproic acid. Though plasminogen enriched clots were used for this experiment precisely similar results have been obtained when clots containing different plasminogen concentrations were used. These findings offer powerful support to the concept that plasma thrombolytic activity is related to its content of plasminogen activator and that free plasmin activity plays little or no part in thrombolysis.

Evidence from in vivo experiment

In vitro experiments, such as have been described here, may not be directly applicable to the *in vivo* state, since changes in plasma composition may alter the experimental conditions. The biochemical changes occurring in man as a result of massive and prolonged streptokinase infusion have been described elsewhere (24), but two problems, relevant to the application of the present findings to *in vivo* circumstances, are considered in this communication.

Plasmin assays made in 17 patients (total of 88 plasma samples), receiving streptokinase therapy, revealed no evidence of greater plasmin release *in vivo* than would have been anticipated from *in vitro* plasma assay. Sufficient streptokinase was infused to activate all the plasma plasminogen within a one to two hour period and then maintain the plasminogen concentration at zero for the remainder of the treatment period. Plasmin activity was only detectable for the first two to three hours of the treatment period and peak values in the individual patient were of short duration. Assay values found were always less than 10 per cent and usually less than 5 per cent of the potential activity (total conversion of plasminogen to plasmin would equal 100 per cent). Indeed it is possible that these values may have been even lower, as the plasma manipulations, required for assay purposes, may have resulted in a spurious increase in free plasmin assay values.

Figure 6 illustrates the effect of the various fibrinolytic moieties found in plasma during streptokinase therapy upon an assay system of clots

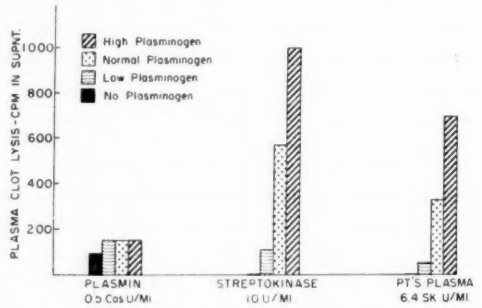


FIG. 6. EFFECT OF FIBRINOLYTIC MOIETIES PRESENT IN STREPTOKINASE (SK) ACTIVATED PLASMA UPON RADIOACTIVE CLOTS

The right hand column shows the thrombolytic activity of plasma drawn from a patient receiving streptokinase therapy upon clots of different plasminogen content. The general similarity of this pattern of activity to that displayed in the center column (the action of streptokinase upon identical clots) and its dissimilarity to the pattern of activity found with plasmin (shown on the left) is obvious. Furthermore the quantity of plasmin used was much greater than can ever be demonstrated in plasma.

containing four different plasminogen concentrations. On the left is shown the effect of the maximal plasmin activities found *in vivo* (after acidification of the plasma); in the center, the effect of streptokinase (10 units per ml. buffer) on the same assay system; and on the right, the effect of a plasma sample withdrawn from a patient three hours after the start of treatment. This patient received tracer doses of isotopically labeled streptokinase for the purpose of determining plasma streptokinase concentrations and at the time that the sample was drawn the value for free streptokinase units per ml. plasma was 6.4. The center and right hand columns show identical ratios of activity on clots of different plasminogen content and it is apparent that *in vivo* and *in vitro* clot lysis mechanisms are similar in nature.

DISCUSSION

Human plasminogen preparations, when treated with streptokinase, develop both activator and proteolytic activity. However a marked dissociation is apparent between the streptokinase/plasminogen ratios required for the maximal development of these two activities. With a fixed quantity of plasminogen, the addition of a relatively limited amount of streptokinase will induce full proteolytic activity but a restricted degree of activator activity. Increasing concentrations of streptokinase cause an increase of activator activity approximately related to the log concentrations of the added streptokinase, but proteolytic activity does not increase and later, when activator activity is still rising, commences to decline.

In a like manner, the addition of an excess of streptokinase to plasma produces both a proteolytic and also an activator effect. The proteolytic activity is almost completely inhibited by plasma antiplasmin, but the activator effect (assayed on the bovine fibrin plate, which is not sensitive to the action of streptokinase alone) persists, even when plasminogen is no longer demonstrable as a consequence of its activation. These findings are identical with those obtained by study in patients, in whom activator formation remained unimpaired after plasma plasminogen concentrations had been maintained at zero or virtually zero levels for 30 hours by streptokinase infusion (24).

Though plasminogen activation occurs as a two stage process (20, 21) and requires proactivator as an essential component for its completion, clots containing plasminogen also contain a sufficient amount of proactivator to allow of their lysis by streptokinase alone. Experiment showed that clots contained in streptokinase buffer solution lysed in a manner comparable with clots suspended in streptokinase treated plasma. Thus the lysis of clots in streptokinase treated plasma, by activation of their contained plasminogen, could occur either through the direct action of preformed activator or be caused indirectly by streptokinase alone. Insufficient evidence is available to evaluate the relative importance of these mechanisms, but conceivably, under *in vivo* conditions, one may be more important than another.

These results offer no evidence to support the view that digestion of clots or thrombi by the direct action of circulating plasmin is of practical importance. First, the appearance of plasmin, during and after the process of complete activation of plasminogen *in vitro*, is transient and its concentration is very low. Second, assay of plasma from patients receiving streptokinase therapy has shown that no greater quantity of plasmin is formed *in vivo* than would have been expected from predictions based on *in vitro* plasma assay. Third, *in vitro* experiment showed that plasmin digested clots, of differing plasminogen content, at a uniform, relatively slow rate and that if used in concentrations similar to those found in plasma its effects were negligible. Fourth, plasma drawn from patients, receiving streptokinase therapy, lyses clots of varying plasminogen content in a manner identical to that of plasma to which streptokinase has been added *in vitro* and differently from that seen with plasmin. Lastly, the addition of a specific inhibitor of activator, epsilon amino caproic acid (16), in a concentration insufficient to inhibit plasmin, abolishes plasma thrombolytic activity.

On the other hand, there is strong evidence that thrombolysis results from the direct or indirect activation of thrombus plasminogen. First, direct assay for plasminogen, within the thrombus, disclosed the presence of sufficient plasminogen to account for the observed results. Second, with a fixed activator concentration contained in either buffer or plasma, thrombolysis rates were linearly related to the logarithm of the clot plasminogen concentration. Third, with clots of fixed plas-

minogen concentration, thrombolysis rates were linearly related to the logarithm of the activator concentration. Fourth, assays for activator were highly correlated with thrombolysis rates of clots containing normal amounts of plasminogen. Lastly, identical patterns of thrombolysis were produced by comparable plasma specimens whether these were prepared *in vitro* or drawn from streptokinase treated patients and in both cases low concentrations of epsilon amino caproic acid completely inhibited thrombolytic activity.

These experiments demonstrate that the chief and primary mechanism of plasma thrombolysis involves the activation of intrinsic clot plasminogen with resulting autodigestion of the thrombus and that the secondary mechanism of thrombolysis by exogenous plasmin action appears to be relatively unimportant. Therefore we propose that the word thrombolysis be used to designate the entire phenomenon by which preformed thrombi are lysed and which for practical purposes describes a complex series of events inclusive of a final step involving the local action of plasmin confined within the substance of the thrombus. The word fibrinolysis will then be restricted to its original meaning and will designate the action of a proteolytic enzyme acting primarily upon a fibrin substrate, but also acting secondarily upon such other substrates as are susceptible to its action.

The necessity for such a clarification of nomenclature is emphasized by the finding that the addition of urokinase to plasma produces similar results to those reported with streptokinase; a finding that also serves to establish the general importance of these mechanisms to the body economy. These observations exemplify a new biological principle of perhaps considerable significance as they constitute the first demonstration of how an enzyme of wide proteolytic activity (plasmin) may *in vivo* retain its full spectrum of activity, but through local mechanisms have its specificity virtually limited to one substrate (fibrin). Indeed, unless such a mechanism existed, phenomena causing plasminogen activation might well be attended by catastrophic consequences to the organism.

SUMMARY

1. The respective roles of plasminogen activators and plasmin in human thrombolytic mechanisms have been investigated. Quantitation of thrombolysis was accomplished by the use of I^{131} labeled human plasma clots. Test systems using labeled clots containing varying concentrations of plasminogen permitted differentiation between the actions of plasminogen activators and plasmin itself.

2. Studies on streptokinase treated patients confirmed that the conclusions drawn from *in vitro* test systems were applicable to the *in vivo* state.

3. It was demonstrated that the chief and primary mechanism of plasma thrombolysis involved the activation of intrinsic clot plasminogen with resulting autodigestion of the thrombus. The secondary mechanism of thrombolysis by exogenous plasmin action appeared to be relatively unimportant.

ACKNOWLEDGMENTS

It is a pleasure to record our indebtedness to Miss Geraldine Nelson and Mr. Charles Chisenhall for technical assistance. We also wish to thank those physicians at the Barnes and Jewish Hospitals who referred patients for treatment.

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THE MAINTENANCE OF A SUSTAINED THROMBOLYTIC STATE IN MAN. I. INDUCTION AND EFFECTS *

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Streptokinase, a product of hemolytic streptococcal metabolism, is a highly effective activator of human plasminogen. Plasmin, the enzyme formed following plasminogen activation, exerts powerful proteolytic and fibrinolytic effects at neutral hydrogen ion concentrations, which *in vivo* mediate the mechanisms of physiological fibrinolysis. Consequently, hopes have been entertained that the intravenous injection of streptokinase, or of streptokinase-activated plasmin, might prove to be of benefit in the therapy of human thromboembolic disease.

Animal experiment, despite the lessened effectiveness of streptokinase as an activator of animal plasminogen and the difficulties of species variability, has yielded striking findings. The intravenous injection of very large doses of streptokinase has been shown to produce lysis of artificially produced thrombi in the arteries of dogs (1) and in the veins of rabbits (2), to cause the clearance of peritoneal exudate evoked by trauma (3) and more recently to prevent or remove fibrinoid lesions secondary to the generalized Shwartzman response (4). These findings appear to be of unequivocal significance but the precise mechanism of their production has been uncertain.

Human experiment has so far been of limited scope. Tillett, Johnson and McCarty (5) were the first to demonstrate that the intravenous injection of relatively small doses of streptokinase would induce a short-lived fibrinolytic state in man. Their findings were, of necessity, exploratory as the streptokinase preparations available to them were impure and caused pyrogenic and hypotensive effects. Similarly investigations made using streptokinase-activated plasminogen have been limited by the clinical toxicity (6, 7) and the low potency and instability of the available preparations.

However, earlier experimental limitations have now largely disappeared as the development of streptokinase purification methods (8) has resulted in the availability of highly purified streptokinase, which is virtually devoid of toxicity to man (9). Furthermore, better understanding of the role of streptokinase antibody in the inhibition of the fibrinolytic response to streptokinase infusion and a knowledge of plasma streptokinase clearance rates under a variety of immunological circumstances (10) now allows of considerable variation in and control of experimental conditions.

The preceding paper (11) cites evidence to show that the chief and primary mechanism of thrombolysis involves the diffusion or adsorption of plasminogen activator to the thrombus, activation of intrinsic thrombus plasminogen and thrombolysis. The secondary mechanism of direct thrombus digestion by extrinsic plasmin is of little quantitative importance. These findings on mechanisms causing thrombus dissolution have important therapeutic as well as physiologic implications. The use of streptokinase as a plasminogen activator requires either that dosage be regulated to maintain maximum plasmin concentrations or that dosage be regulated to obtain high levels of activator concentration. The rapid fall of plasma plasminogen concentration occurring in the presence of high levels of circulating activator renders the two states mutually exclusive. For the reason cited above we have regulated streptokinase dosage to obtain high levels of activator concentration.

Hitherto no distinction has been made between the two mechanisms of clot lysis described above. We have proposed (11) and have used, in this communication, the term thrombolytic to designate the process of thrombolysis, *i.e.*, the lysis

* This work was supported by grants from the National Heart Institute (H3745), United States Public Health Service, Bethesda, Md., and Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

of a preformed clot,[1] a phenomenon mainly dependent on the concentration of surrounding plasminogen activator. We have restricted the use of the term fibrinolytic to its original meaning to indicate the action of a proteolytic enzyme acting primarily upon a fibrin substrate but also acting upon such other substrates as are susceptible to its action.

It is the purpose of the present communication to describe the physiological and biochemical changes resulting from the intravenous infusion at high dosage of streptokinase in man. Since the direct toxic effects of streptokinase were minimal, the study constitutes an investigation of the biological effects of an intense and prolonged thrombolytic state in the human. The results indicate that, at least under restricted circumstances, this state is tolerated without deleterious consequences and may be applied to the treatment of thromboembolic disease in man. The clinical effects of such a sustained thrombolytic state are described in an accompanying article (12).

MATERIALS AND METHODS

The observations reported in this communication were made on 50 hospitalized patients suffering from thromboembolic disease, who received streptokinase therapy[2] (12). Blood specimens were collected into oxalate tubes, 0.8 ml. of 5 per cent potassium oxalate being used for 10 ml. of blood. One ml. of blood was collected into a plain 75 × 6 mm. tube and incubated at 37° C. for observation of the whole blood fibrinolysis rate. Specimens for platelet counts were collected in ethylenediaminetetraacetic acid (EDTA) tubes. Sedimentation rates and hematocrit determinations were performed in standard Wintrobe tubes. Assay methods for all components of the plasminogen plasmin system and for fibrinogen[3] were as previously described (11).

Streptokinase was isotopically labeled and its plasma concentration determined in patients by previously described methods (10). Quantitative paper electrophoretic determinations were made with a Spinco[4] R apparatus using standard staining and elution methods (13).

Antiplasmin assay. The antiplasmin activity of plasma was assayed by incubating the plasma with spontaneously activated human plasmin containing 3.5 casein units per ml. (14) and determining the degree of inhibition produced. Plasmin aliquots (0.5 ml. containing 1.75 casein units) were incubated with 0.2 ml., 0.1 ml. and 0.05 ml. of the test plasma for 15 minutes at 37° C. Each sample was then assayed for residual plasmin by the casein method and the result expressed as percentage inhibition of the original plasmin solution. Though the inhibition of plasmin by plasma is approximately related to the log plasma concentration, we have not calculated absolute antiplasmin values since these values depend, to a degree, upon the conditions of measurement.

Urokinase lysis time. This test facilitates differentiation between the presence of streptokinase antibody and plasmin inhibitors in plasma. Two-tenths ml. of plasma plus 0.1 ml. urokinase (0.5 mg. per ml.)[5] were clotted with 0.1 ml. thrombin (20 units per ml.) and observed for plasma fibrinolysis at 37° C.

One stage prothrombin times were determined by the method of Quick (16) using rabbit brain thromboplastin.[6] The same batch of thromboplastin was used throughout and normal one stage times were 15.2 ± 1.1 seconds.

Two stage prothrombin assays were determined by the method of Ware and Seegers (17) modified by the use of dextran (final concentration 0.6 per cent) rather than acacia in the incubation mixture. Standard plasma[7] was used as a control and gave an assay reading of 275 prothrombin units per ml.

Accelerator globulin (*AcG*) concentrations were routinely determined by the method of Deutsch and Schaden (18) and in some patients the method of Ware and Seegers (19) was also employed to check the findings. Cognizance was taken of the increased antithrombin values shown in some plasmas and its effect upon the AcG values was eliminated by appropriate specimen dilution.

Antithrombin assay. The clotting time of a patient's plasma by the addition of a standard thrombin solution was used as a measure of antithrombin activity. Parke, Davis thrombin was dissolved in a mixture of equal parts of glycerol and saline to a concentration of 100 U per ml. and stored at −20° C. Immediately before use this thrombin was diluted with saline 1 : 10. To 0.1 ml. of the patient's plasma was added 0.3 ml. thrombin titration mixture (20). One-tenth ml. thrombin, 10 U per ml., was blown into the mixture and the clotting time

[1] This usage corresponds to the literal meaning and original derivation of the word (Oxford English Dictionary) and its use does not imply specific reference to pathological states where intravascular thrombi may vary in composition.

[2] Observations on a preliminary series of 36 patients (15) although used to develop the present methods are excluded from this report.

[3] Since this method depends on complete conversion of fibrinogen to fibrin, some values quoted later may be in error, because until the presence of antithrombin activity was detected in certain of our plasma samples, only a one hour precipitation time was used. Later highly reproducible duplicate assays were obtained by using a 10 hour precipitation period.

[4] Spinco/Beckman Instruments, Berkeley, Cal.

[5] Kindly supplied by Dr. J. Ploug, Leo Pharmaceuticals, Copenhagen.

[6] Permaplastin, C. W. Alban Co., St. Louis, Mo.

[7] Diagnostic plasma, Warner-Chilcott, Morris Plains, N. Y.

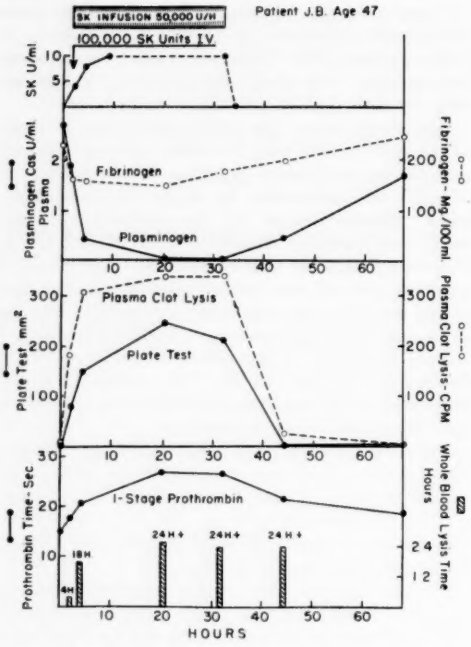

FIG. 1. SERIAL BIOCHEMICAL DETERMINATIONS IN A PATIENT RECEIVING STREPTOKINASE THERAPY

The infusion and the thrombolytic state were of 30 hours' duration.

noted. The thrombin titration mixture was added to give a sharper end point and shorter clotting times; furthermore our results were far more reproducible by this method than without the thrombin titration mixture. Clotting times of normal plasmas performed in this manner were 9.9 ± 1.1 seconds.

Transaminase (S-GOT) was determined by the method of Karmen, Wróblewski and LaDue (21).

Method of streptokinase administration. The streptokinase preparations [8] used, assayed at 600 to 700 streptokinase units per μg. nitrogen, were biophysically homogenous and though showing evidence of nonhomogeniety on immunochemical analysis were devoid of other than streptokinase activity.

The plasmas of patients were assayed for their total content of streptokinase inhibitory constituents (antibody and antiplasmin) by means of the predicted dose test (22). Since the purpose of the initial priming dose of streptokinase was to induce a rapid fall of plasma plasminogen, this dose was calculated using a 20 minute test end point rather than the 120 minute end point previously described (22); the effect of this change was to approximately double the streptokinase concentration reading per ml. plasma. Thus the initial streptokinase dose required for each patient was calculated by multiplying the number of streptokinase units required to produce plasma fibrinolysis in 1 ml. plasma in 20 minutes by the patient's calculated plasma volume. This initial dose varied between 35,000 and 1,500,000 units for individual patients and was dependent chiefly upon the patient's plasma antibody level; it was injected intravenously over a 10 to 40 minute period. Samples withdrawn following this procedure invariably showed brisk whole blood fibrinolysis. Expressed as a frequency distribution, the initial dose of streptokinase employed was less than 50,000 units in 35 patients, 50,000 to 100,000 units in 19 patients, 100,000 units to 1,000,000 units in 19 patients and over 1,000,000 units in three patients. Analysis of this frequency distribution against a similar one (22) showed that our patients needed substantially less streptokinase as an initial dose than had been previously calculated (χ^2 equals 17.6 for five degrees of freedom and $0.01 > p > 0.001$).

Infusions were usually given into a foot vein and the requisite constant delivery rate maintained by the use of a mechanical syringe driver.[9] Streptokinase dissolved at appropriate concentrations in normal saline was administered at the rate of 7 ml. per hour; these solutions maintained at 25° C. were stable for more than 24 hours. The infusion schedules were based on a study of plasma streptokinase clearance rates made with isotopically labeled streptokinase (10) and checked by determination of actual plasma streptokinase concentrations, using similar methods. In practice, it was found that patients receiving an initial priming dose of streptokinase of 50,000 units or less required a sustaining infusion at the rate of 35,000 streptokinase units per hour. Those receiving an initial dose of 50,000 to 100,000 units required a sustaining infusion at the rate of 50,000 units per hour, and those receiving an initial dose of over 100,000 units required 100,000 to 150,000 units per hour. The mean plasma streptokinase concentration obtained by sampling during the infusion periods was 8.5 streptokinase units per ml. plasma with a range of three to 19 streptokinase units per ml. plasma (21 observations made with isotopically labeled streptokinase in five patients). These figures and the consistent biochemical data, quoted later, indicate that the infusion schedules provided effective concentrations of streptokinase and plasma activator throughout the infusion period. The duration of the infusions varied from six to 48 hours.

RESULTS

Changes in fibrinolytic moieties during streptokinase therapy

Figure 1 illustrates biochemical changes following the injection of 100,000 units of streptokinase given as a priming dose and 50,000 streptokinase

[8] Kindly supplied by Dr. J. Ruegsegger, Lederle Laboratories Division of American Cyanamid, Pearl River, N. Y.

[9] Bird Kymograph Driver, Phipps and Bird, Inc., Richmond, Va.

units per hour for 30 hours as a sustaining infusion; these changes were typical of those found in this series. The patient, a 47 year old male, suffering from pulmonary embolism, also received tracer doses of isotopically labeled streptokinase in both the priming injection and in the sustaining infusion for the purpose of measuring plasma streptokinase concentrations. So as to limit the quantity of administered radioactivity to 30 μc., the isotopically labeled streptokinase infusion was given only over the first eight hour period and plasma streptokinase concentrations beyond this period have been calculated (interrupted line in Figure 1).

The priming dose of streptokinase, though largely cleared from the plasma in combination with antibody, sets in train a number of biochemical changes, which rapidly become complete and stabilize under the action of the sustaining infusion. As plasma streptokinase concentration increased, plasminogen was rapidly and completely activated to plasmin and the plasma plasminogen concentration fell to zero or trace levels. The relatively high and constant plasma streptokinase concentration, maintained throughout the infusion period, caused immediate activation of plasminogen entering the circulation and as a consequence plasma plasminogen concentration remained at near zero or trace levels. Despite the complete activation of available plasminogen to plasmin, assay for this enzyme in the plasma of patients who received streptokinase has revealed that plasma inhibitory mechanisms were so effective and rapid in action that only minimal proteolytic activity could be detected, *i.e.,* usually less than 5 per cent of the potential activity (11). Such residual proteolytic activity, highest at the start and during the first few hours after the start of the infusion, caused among other changes (*vide infra*) an initial drop of plasma fibrinogen concentration (approximately 30 per cent). However, following the initial drop, the level stabilized and indeed, in some patients, actually slowly increased as the infusion was continued.

Concomitantly with the initial rise of plasma streptokinase concentration, intense plasma thrombolytic activity developed. Thrombolytic activity, as measured by both the rate of digestion of isotopically labeled clots and by the fibrin plate technique, remained greatly elevated throughout the duration of the infusion and declined abruptly upon its cessation. That this effect is due to the presence of plasminogen activator rather than to the presence of the enzyme plasmin in the patient's plasma has been demonstrated by activating or removing plasminogen from the assay systems. Either heating the fibrin plate to denature plasminogen or washing the isotopically labeled clots to elute plasminogen results in test systems insusceptible to the action of patient's plasma but susceptible to the action of plasmin (11).

Whole blood fibrinolysis times reflect the composite biochemical changes shown in Figure 1 and though enhanced by the presence of plasmin, depend primarily upon the rate at which plasminogen is being activated within the formed clot (23), a phenomenon dependent on the plasminogen and activator concentrations of the plasma from which the clot was formed. The specimen drawn 30 minutes after the start of the infusion contained 60 per cent of its original plasminogen concentration and the resulting clot, though somewhat deficient in plasminogen, lysed in four hours under the influence of plasminogen activator present at this time. At three hours, though the plasminogen activator concentration was approximately double that in the previous specimen, clot lysis did not occur for 18 hours as the clot was formed from plasma containing only 10 per cent of its normal plasminogen content. At 20 and 30 hours, though plasminogen activator was still present in high concentration, neither clot lysed during the period of observation as they were formed from plasminogen-free plasma. The failure of clot lysis at 45 hours (15 hours after the cessation of the infusion) was due to lack of plasminogen activator, since this material disappears from the plasma two to four hours after the termination of the infusion. Stress is laid upon the phenomenon of whole blood fibrinolysis as it has implications important to the clinical safety of the induced thrombolytic state.

Figure 1 shows that the patient developed a coagulation anomaly evinced by a rise in the one stage prothrombin time from a normal of 15 seconds to a high of 26 seconds at which level the prothrombin time stabilized until it returned to normal after termination of the infusion. All patients receiving streptokinase have developed a similar anomaly, though to varying degree, and its

TABLE I

Changes in fibrinolytic moieties during streptokinase therapy *

Time of sample . . .	0	30 mins.	2–3 hrs.	6–8 hrs.	14–20 hrs.	22–27 hrs.	28–32 hrs.	8–12 hrs. after infusion
Sample no.	0	I	II	III	IV	V	VI	
Plate test, *mm.*²	0	142±108 (393–0)	241±95 (440–133)	230±84 (425–144)	220±95 (420–85)	194±81 (377–101)	156±56 (246–75)	0
Fibrinogen, *mg./100 ml.*	334±86 (475–153)	278±71 (403–144)	221±93 (412–103)	211±83 (371–84)	183±76 (334–67)	204±89 (350–67)	205±70 (344–104)	240±51 (334–146)
Plasminogen, *cas. U/ml.*	2.46±0.92 (4.00–1.08)	1.26±0.73 (2.87–0.17)	0.35±0.43 (1.52–0)	0.13±0.14 (0.48–0)	0.22±0.27 (1.0–0)	0.11±0.16 (0.54–0)	0.10±0.08 (0.29–0)	1.08±0.53 (2.16–0.20)
Clot lysis, *cpm in supnt.*	0	190±118 (398–5)	381±310 (1,000–80)	354±155 (570–99)	451±247 (805–100)	288±171 (583–75)	277±104 (410–100)	0
Whole blood fibrinolysis, *hours*	24+	3 (0.5–24+)	3 (0.5–24+)	5 (3–24+)	14 (6–24+)	21 (18–24+)	23 (18–24+)	24+

* Biochemical changes observed in 21 patients. (Mean and standard deviations are given with range of observations in parentheses.) Ten patients in this group received supplemental hydrocortisone and the data given for plate test, fibrinogen, plasminogen and radioactive clot lysis includes these patients. However the data given for whole blood fibrinolysis was that obtained in the nonsteroid treated group.

pathogenesis with the means for its control are discussed in a later section.

Table I embodies data obtained from analyses made on 128 blood samples drawn from 21 patients who received continuous streptokinase treatment for 30 hours. Biochemical data obtained from 29 other patients differed in no respect from that recorded in Table I, but since some of the findings referred to infusions of differing duration and in others serial sampling was more widely spaced, the data have not been incorporated. Intravenous hydrocortisone was administered before and during the treatment to 10 of the 21 patients, but since analysis showed no significant differences ($p > 0.1$ in all instances) between the steroid and the nonsteroid treated groups, the data for plasminogen, fibrinogen, fibrin plate test and isotopic clot assay given in Table I represent the combined values from both groups. However hydrocortisone administration did affect other phases of the thrombolytic state and the times for whole blood fibrinolysis given in Table I refer only to patients not given steroids.

It is apparent from the mean values for the biochemical determinations and their comparatively narrow standard deviations (Table I) that the injection of an appropriate priming dose and a sufficient sustaining infusion of streptokinase will cause predictable and highly reproducible biochemical changes in patients. Though the dosage schedule described earlier was initially used in all patients, in some 10 per cent of them the infusion rate was increased if analysis of samples drawn within the first six hours showed an insufficiently intense thrombolytic state. The indications for increasing the infusion rate were either an insufficiently lowered plasminogen concentration or a low value for the isotopic clot dissolution test; a dose increase of 30 to 50 per cent was always sufficient to induce biochemical changes of the desired degree. Though the mean fall of fibrinogen concentration averaged 30 per cent of the initial concentration, sometimes fibrinogen values fell to as low as 100 mg. per 100 ml. plasma. However, for reasons discussed earlier, in footnote three, it is probable that some of these determinations were misleadingly low and the actual fall in plasma fibrinogen that occurred may be less than stated here. All blood specimens, withdrawn from the patients, clotted in a macroscopically normal manner and the clots were of a sufficiently firm consistency to resist inversion of the specimen tube. Thus, despite the apparent magnitude of the plasma fibrinogen fall in some patients, treatment was never terminated on this account.

The activation of human plasminogen by streptokinase takes place by a two stage reaction (24, 25). First streptokinase reacts with a plasma component (termed proactivator) and the resulting activator reacts with plasminogen enzymati-

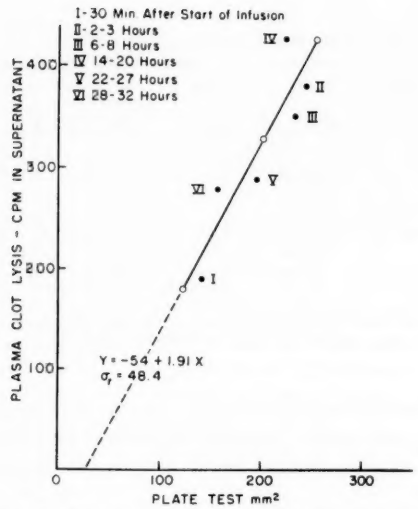

FIG. 2. PLASMA CLOT LYSIS (CPM) PLOTTED AGAINST FIBRIN PLATE TEST AT VARIOUS TIMES DURING STREPTOKINASE (SK) INFUSION

A constant ratio between the two tests, the one being sensitive both to the presence of streptokinase-plasma activator and to streptokinase, while the other was sensitive only to the former indicated that activator depletion did not occur ($r = 0.88$ and $p = 0.02$).

cally. Proactivator has yet to be isolated or characterized but present evidence suggests that it may be closely associated with or a derivative of plasminogen (26). Since in our patients serum plasminogen concentration has been held at zero or near zero levels for many hours, proactivator concentration could also have fallen sufficiently to impair effective plasma thrombolytic activity. Plasma proactivator concentrations were not assayed directly as the available methods are unsatisfactory but observations on plasma activator concentrations yielded important indirect information. Whereas the isotopic clot dissolution test was susceptible to both the action of streptokinase and to streptokinase induced plasma activator, the fibrin plate test was susceptible only to the latter substance (11). Thus a marked decline in plasma proactivator concentration would have caused an alteration in the ratio of the plasma activities assayed by the two tests. Figure 2 shows (data taken from Table I) that the ratio of plasma activities upon these two tests remained constant regardless of the time after the start of the infusion at which the plasma was drawn. The points lie randomly (with regard to the time after the start of the infusion) along a regression line ($r = 0.88$ and $p = 0.02$) and analysis of variance disclosed no evidence of nonlinearity. This observation indicates that the effectiveness of the plasma thrombolytic state was not impaired by lack of proactivator.

Platelet counts

Serial platelet counts were performed on six patients during the course of therapy. The initial dose of streptokinase varied between 60,000 and 800,000 streptokinase units (100 to 1,300 μg. protein nitrogen), being assessed individually for each patient on the basis of plasma assay (22) and being injected intravenously over a 15 minute period. The patients selected showed evidence of either a moderate or a high degree of immunization against streptokinase and the 30 hour sustaining infusion was run at a rate of 200 to 400 μg. streptokinase per hour.

Inspection of the platelet counts failed to show any disturbance in their values as a consequence of the streptokinase administration. The actual counts were: at start $243{,}000 \pm 59{,}000$; 30 minutes after start $223{,}000 \pm 78{,}000$; two to three hours after start $252{,}000 \pm 73{,}000$; eight hours after start $309{,}000 \pm 92{,}000$; 12 hours from start $342{,}000 \pm 147{,}000$; 30 hours from start at termination of infusion $280{,}000 \pm 112{,}000$; 24 hours after the termination of the infusion $273{,}000 \pm 98{,}000$ (all values for p exceed 0.1).

Capillary fragility

Capillary fragility was estimated by the suction cup method (27), at two to six hourly intervals in seven patients receiving 30 hour streptokinase therapy. No change of capillary fragility was found and in no instance were petechial hemorrhages produced at suction pressures of less than 50 mm mercury.

Serum proteins

In five patients receiving 30 hour streptokinase therapy one serum sample drawn before the start of therapy, four drawn during its course and one after its termination were examined by paper electrophoresis. Albumin, alpha$_1$, alpha$_2$, beta and gamma globulin fractions were estimated by stain-

TABLE II

Effect of streptokinase therapy upon one stage prothrombin times *

	0	30 min.	2–3 hr.	6–8 hr.	18 hr.	26 hr.	30 hr.	+6–12 hr.
Clotting time in seconds ±S. D.	15.9±1.7	19.8±4.2	26.5±11.6	29.9±11.7	25.4±3.0	24±2.5	23.9±1.9	20.2±4.3
Range	13–19	16–31	21–>60	20–>60	20–28	22–27	22–27	15–25
				Steroid treated patients				
Clotting time in seconds ±S. D.	15.3±1.2	16.2±1.2	21.7±5.1	23.8±2.2	22.0±2.7	20.0±1.7	21.8±1.0	19.8±3.4
Range	14–17	15–18	17–30	21–26	21–28	18–22	21–23	17–23

* The effect of supplemental steroid therapy was to diminish the rise in one stage prothrombin time that occurred. Analysis showed that the significance of the difference between the observations recorded over the first eight hour period was p = 0.02.

ing the strips and eluting the fractions. No significant alterations of the serum protein fractions were found.

Serum transaminase (SGO-T)

Serial assays were run in nine patients receiving therapy. No deviations from the base level occurred.

The coagulation defect

A coagulation defect, demonstrable in every instance by a rise in the one stage prothrombin time, developed in all patients receiving massive streptokinase dosage. Moreover, some of these patients, at or after the time that the one stage prothrombin time was most grossly elevated, experienced clinically evident episodes of untoward bleeding from various anatomic sites. Table II, upper section, shows serial one stage prothrombin times found in treated patients and the hemorrhagic complications are described in the next section on the hemorrhagic diathesis.

One stage prothrombin times commenced to rise immediately following the injection of the priming dose of streptokinase during the initial phase of plasminogen activation. Peak values were usually attained at six to eight hours after which they frequently declined to stabilize at a lower level during the remainder of the infusion period and recovery to normal values for the test took eight to 24 hours after its end. A few patients experienced such a great elevation of their one stage prothrombin times in the first four to eight hour period that treatment had to be terminated. In these patients, following the cessation of the streptokinase infusion, a decline toward more normal one stage values was apparent within four hours and virtually normal values were found 16 to 30 hours later.

The pathogenesis of the rapidly developing clotting defect is displayed in Figure 3. The defect illustrated, the most extreme encountered in our series, developed in a 72 year old male receiving his first streptokinase treatment. Shortly after the start of the infusion the one stage prothrombin time rose rapidly and the patient's plasma developed an intense degree of antithrombin activity,

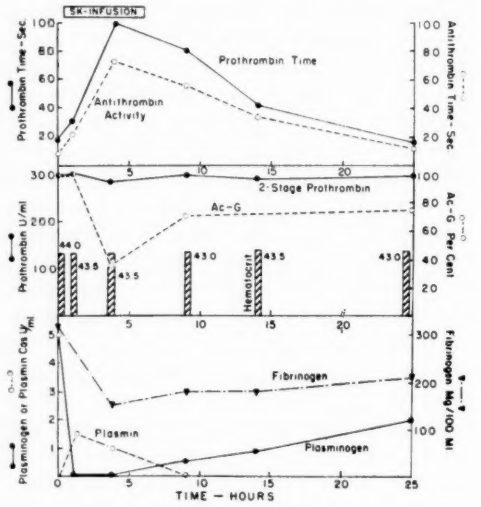

FIG. 3. EXTREME COAGULATION DEFECT DURING STREPTOKINASE THERAPY

Deleterious clinical complications were absent despite the severity of the coagulation defect that necessitated the termination of streptokinase treatment.

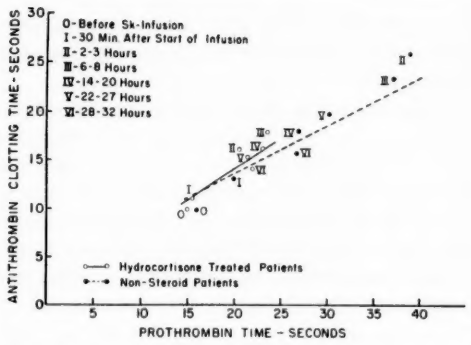

FIG. 4. RELATIONSHIP BETWEEN PROTHROMBIN TIME AND ANTITHROMBIN CLOTTING TIME AT VARIOUS TIMES DURING STREPTOKINASE (SK) INFUSION

One stage prothrombin times were closely correlated at all stages during the infusion with determinations of plasma antithrombin activity. For nonsteroid treated patients $r = 0.93$, $p < 0.001$. Significant differences did not exist ($p > 0.1$) between steroid and nonsteroid treated patients.

which paralleled the prothrombin time rise. Coincidentally a fall of plasma accelerator globulin content was found but quantitatively this was of insufficient degree to have exerted important effects upon the prothrombin time. Whole blood fibrinolysis remained brisk throughout the infusion period and noteworthy was the presence of a high level of circulating plasmin, some fivefold greater than that normally found under these circumstances (11). Despite the development of these gross abnormalities in plasma coagulation moieties, plasma prothrombin concentrations (determined by the two stage test) remained unchanged and whole blood clotting time determinations (made in glass tubes) showed only small and probably insignificant variation. Clinically, the patient showed excessive oozing of blood from venipuncture sites and despite the eventual formation of apparently firm clots, ecchymoses developed in these areas. Serial hematocrit determinations remained unaltered, blood specimens formed normally retracted clots, changes in platelet count did not occur and determinations of capillary vessel fragility remained within normal limits. Repeated examination of stool and urine specimens were negative for blood and other untoward signs or symptoms were absent.

Investigation of other patients has shown identical qualitative changes in the pattern of the coagulation defect, but usually these were of a much lesser degree. Serial assays for accelerator globulin have shown that the mean plasma content (expressed in per cent) was 56 ± 18 (range 31 to 100 per cent) at the time of greatest depression, which occurred between two to six hours after the start of the infusion. This fall was normally insufficient to have altered the one stage prothrombin time more than one to two seconds. Certain patients received supplemental hydrocortisone, but analysis revealed no difference ($p > 0.1$) with respect to the degree of change in accelerator globulin depression in the two series. However a very striking correlation was present between the rising one stage prothrombin time and the development of increasing plasma antithrombin activity ($r = 0.93$ $p < 0.001$); this relationship is illustrated in Figure 4. Such an association offers highly suggestive evidence that the abnormality of the one stage prothrombin time found in our patients resulted from the development of plasma antithrombin activity and further evidence in favor of this contention will be developed.

The effect of hydrocortisone administration on the induced fibrinolytic state and the clotting defect

During the later stages of the investigation all patients received supplemental intravenous hydrocortisone. Twenty mg. hydrocortisone was given prior to the injection of the first streptokinase dose and steroid administration was continued at the rate of 10 mg. per hour for the first eight hours of the infusion and thereafter at the rate of 5 mg. per hour until its termination. This schedule was sometimes varied and some patients received only a total of 100 mg. hydrocortisone by the intravenous route, maintenance of steroid therapy being accomplished by the use of intramuscular cortisone preparations. Differences were not apparent between the results of the two schedules of steroid administration and the combined patient group is referred to collectively as steroid treated.

Mention has been made earlier that supplemental steroid treatment caused no change in assays for plasminogen concentration, fibrin plate test, or isotopic clot dissolution assays, all of which are shown in Table I. However, despite the fact

that fibrinogen falls in the two groups (steroid and nonsteroid treated) were similar as were decreases in plasma accelerator globulin ($p > 0.1$ in each instance), striking differences were apparent in the degree of whole blood fibrinolysis recorded. Of 30 blood samples withdrawn from 10 steroid treated patients during the first eight hours of steroid treatment, 20 failed to lyse over a 24 hour observation period; of 48 samples drawn from nonsteroid treated patients during the same period, only six failed to lyse within the same time. Analysis either on the basis of lysis times between the groups or on the basis of lysis or no lysis within the period of observation give $p < 0.001$ in each case.

The rise in the one stage prothrombin time following streptokinase treatment was also much reduced by the administration of steroids. The extent of this reduction is given by comparison of the upper and lower sections of Table II: Figure 4 shows a plot of the one stage prothrombin time against the plasma antithrombin activity in the same patients. Analysis of the prothrombin times recorded over the first eight hours of the infusion period between the steroid and the nonsteroid treated groups showed p to be 0.02. Actually the differences were larger than shown, as on account of the development of excessively elevated one stage times, the streptokinase infusion was sometimes stopped early in the nonsteroid treated group. These changes in laboratory observations were associated with a remarkable reduction in the incidence of toxic effects attributable to the streptokinase treatment. Toxic effects will be referred to in the section on the hemorrhagic diathesis.

Test of the two regression lines shown in Figure 4 indicates that they do not differ significantly ($p > 0.1$), though the fit of the line in the steroid treated patients is noticeably inferior to that found with the nonsteroid treated group. This difference is most easily explained by the differing effect, on a proportional basis, of the substantially equal fall of plasma accelerator globulin that occurred in both groups. Whereas in the steroid treated group the accelerator globulin fall was associated with a relatively minor rise of plasma antithrombin activity, in the other group the rise of antithrombin activity was much greater. This concept is supported, but not proven, by analysis of the proportional defect ratio upon the one stage prothrombin time at various times after the start of the infusion.

In summary, the effect of supplemental steroid treatment was to reduce the rate and degree of whole blood fibrinolysis, to restrain the development of plasma antithrombin activity and thus to reduce the elevation of the one stage prothrombin time that would otherwise have occurred. Changes in other coagulation and fibrinolytic moieties secondary to the use of steroids were not detected.

The hemorrhagic diathesis

The development of a mild clinical hemorrhagic diathesis was common in those 34 patients treated without supplemental steroid therapy but was seen in only one patient who did receive steroids. Usually the only manifestation of the diathesis was excessive oozing from venipuncture sites and the subsequent appearance of ecchymotic areas related to these sites, though sometimes hematomatas were formed at other injection sites. Occasionally more serious manifestations of a clinical hemorrhagic nature were apparent and these occasions are classified below in connection with certain predisposing factors.

1) Major operative wounds. Since the first stage of wound healing involves the deposition of fibrin, the induction of a thrombolytic state would be expected to cause fibrin lysis and hemorrhage around and into the wound. Nevertheless, provided that hemostatic mechanisms remain unimpaired, the hemorrhage would be expected to be self-limiting, as fibrin laid down during the thrombolytic state would be lacking in plasminogen and thus be insusceptible to autolysis by plasma activator.

Three patients were treated with streptokinase within 12 to 24 hours of major operative intervention. In two patients the femoral, popliteal and posterior tibial arteries had been exposed and in the third the axillary artery. Though in each instance the operation had failed to improve the limb blood supply, the centripetal portion of each deep and extensive wound had a normal blood supply from collaterals and the centrifugal portion a somewhat diminished one. Within six hours of starting streptokinase treatment, the wound margins became grossly ecchymotic, but

though in each instance they were loosely sutured, only small quantities of blood drained from the wounds, not perceptibly greater in amount than that draining before the start of streptokinase. One patient, just prior to streptokinase therapy, had three large deep wounds inflicted on the arms in an unsuccessful attempt to cannulate a vein. Despite meticulous surgical hemostatic technique, petechial hemorrhages were apparent in the wound margins within four hours of starting streptokinase and large ecchymotic areas were apparent after six hours. However though treatment was given daily for three days and the ecchymotic areas spread to the neck, apparent resolution commenced even while the treatment was being continued.

2) Local disease or trauma. Clinical manifestations of the hemorrhagic diathesis tended to occur, as was not unexepected, at sites of local disease or trauma. A 54 year old male, successfully treated for ileofemoral thrombophlebitis, developed coincidentally with the rapid subsidence of the limb edema a sharp increase in the size of a preexistent knee effusion: A joint tap revealed a bloodstained fluid. This patient also continued to ooze blood from the site of an intramuscular penicillin injection, even after all laboratory evidence of the fibrinolytic state had returned to normal. Three other patients developed large hematomatas at morphia injection sites, an effect probably related to the local pharmacological action of the drug. One patient experienced a small hematemesis following treatment and radiological examination showed a duodenal ulcer. However in view of the time relationship of the treatment and the onset of bleeding, it is possible that the two events were unrelated.

Though the presence of large or relatively large diseased areas appeared to determine the site of bleeding, smaller areas of trauma were without apparent effect. Five patients, prior to and at six hours after the commencement of therapy, were subjected to minor trauma by applying the suction cup of a capillary fragility apparatus at 300 mm. Hg for five minutes on the forearm. In each instance petechial hemorrhages were produced below the cup. They were observed by low power magnification throughout the infusion course: In no instance did the size or number of the hemorrhages increase.

3) Dicumarol® administration. The possible hazard of Dicumarol® effect upon the hemorrhagic diathesis was realized at the start of the investigation, but the exigencies of clinical practice required that eight patients who had received one or more doses of Dicumarol® should be treated with streptokinase. Three of these patients, despite the prior administration of 50 mg. of vitamin K_1 by the intravenous route, developed marked coagulation abnormalities. In the first patient, though excessive oozing accompanied venipuncture, the extent of the coagulation anomaly was only revealed by laboratory study. In the second patient the greatly prolonged one stage prothrombin time (50 seconds at maximum) was associated with minor and transient bleeding from the gums and the demonstration of microscopic hematuria over a two day period. The third patient, a 54 year old male, had been maintained on Dicumarol® for six months, since his first attack of myocardial infarction. At a time when his one stage prothrombin time was 40 seconds, he experienced a further severe attack of myocardial infarction. He was given vitamin K_1 by the intravenous route but six hours after the start of streptokinase his one stage prothrombin time was 60 seconds. Streptokinase was continued for a further 24 hours by which time his one stage time had declined to 32 seconds. However the area around the infusion needle became grossly ecchymotic, his whole arm became greatly swollen (diameter 7 cm. greater than its fellow) and ecchymotic areas appeared around the axilla. Stools and urine were negative for blood, serum bilirubin remained unaltered and values for platelets, bleeding and clotting times were normal. However over the next 10 days his hematocrit fell 10 points, remained depressed for a week and then commenced to rise. Clinically the patient did well and the arm returned to its normal appearance and function.

4) No apparent predisposing cause. Two other patients have shown falls in hematocrit values of six and eight points, respectively, during treatment, without clinical evidence of bleeding, but in each instance this fall could have been consequent upon the primary disease process.

Autopsy evidence

Ten patients died either during or after streptokinase treatment. Clinical protocols and a de-

TABLE III

*Predicted dose values and urokinase lysis times of plasma before and after streptokinase (SK) treatment**

Time of sampling	Before SK	1–2 Months after SK	3–11 Months after SK
Predicted dose test, *SK-U/ml.*	28 ± 15	1,760 ± 1,100†	70 ± 21
Range	(5–55)	(900–4,000)	(20–120)
Urokinase lysis time, *min.*	21 ± 7	18 ± 9	24 ± 10

Antiplasmin assay of immune and nonimmune plasma expressed as per cent inhibition of a standard plasmin solution

Quantity of test plasma	0.2 ml. plasma	0.1 ml. plasma	.05 ml. plasma
Immunized patients (*P.D. 1,760 ± 1,100 SK-U/ml.*) per cent inhibition	70 ± 8	55 ± 6	38 ± 8
Nonimmune patients (*P.D. 32 ± 10 SK-U/ml.*) per cent inhibition	68 ± 7	58 ± 6	39 ± 6

* The upper section illustrates the degree of immunization (on the basis of plasma findings) occurring after streptokinase treatment. The lower section confirms the fact that the data shown resulted from the formation of specific antibody.

† This group is the same as the immunized patients in the lower part of this table.

scription of specific pathological findings are published elsewhere (12), but in no instance was it considered on clinical grounds that the treatment contributed to death. Autopsy permission was refused in the case of a 74 year old woman suffering from myocardial infarction and shock who received only three hours' streptokinase treatment. Three patients died during the course of therapy at seven, 12 and 14 hours, respectively, after its start and a further three patients died one, two and three days, respectively, after its completion. Three more patients died in the two to six week period afterwards. The patients' deaths were so spaced that the likelihood of missing an early or late toxic manifestation of the thrombolytic state was slight. A particular search was made for evidence of untoward old or recent hemorrhage and also for myocardial lesions similar to those described as occurring in rabbits after the injection of crude streptokinase preparations (28). In no instance were pathological findings other than those related to the primary disease observed and in all instances an adequate pathological cause, sufficient to cause death and related to the primary disease, was found.

Immunological consequences

Streptokinase is antigenic to man (10) and the heavy intravenous dosage required to produce an intense and sustained thrombolytic state invariably, or almost invariably, results in rapid immunization of the patient. Immunization may be of such an extreme degree as to prevent, temporarily at least, further induction of thrombolysis by streptokinase should it be required.

However it has been suggested (29) that treatment of the patient with streptokinase activated plasmin may result not only in the stimulation of antibody formation but also cause an early relatively large and important increase in plasma antiplasmin content. This contention, if true, would imply that a state of impaired physiological thrombolytic activity might follow the therapeutic use of sterptokinase or streptokinase activated plasmin.

Table III (upper portion) shows that though plasma obtained from patients one to three months after streptokinase therapy is relatively insusceptible to the action of streptokinase (requiring 40- to 200-fold the original streptokinase concentration to produce a fibrinolytic end point ($p < 0.001$), urokinase retains its former efficacy as a plasminogen activator ($p > 0.1$). This observation suggested that the antiplasmin content of immune and nonimmune plasma was identical. Table III (bottom portion) shows antiplasmin assay made on the one to three month highly immune plasma samples (referred to above) and compared with the antiplasmin content of 17 control nonimmune plasma samples. Since anti-

plasmin combines with plasmin in a nonlinear manner, these assays were conducted using three different concentrations of spontaneously activated plasmin; the differences in antiplasmin values between the two sets of samples were negligible ($p > 0.1$).

Plasma samples (35 in number) were withdrawn from nine patients at intervals from one day to one month after streptokinase therapy. All patients became immunized in a manner similar to that displayed in Table III, but in no instance during the course of the immunization process was the urokinase lysis time significantly altered. These results indicate that changes in plasma constituents following the streptokinase induced thrombolytic state are confined to alteration of specific antibody concentration.

A point of practical importance concerns the duration of persisting high plasma antibody levels following streptokinase therapy, since these, if sufficient, may be a bar to retreatment of the patient. We have followed 17 patients and find that antibody levels commence to decline from their peak at two to three months and the fall thereafter is somewhat variable. Antibody levels had fallen to a sufficient degree to render retreatment practicable in nine of the 17 patients after three months and in the remaining patients retreatment could have been undertaken after six months.

DISCUSSION

Intense plasma thrombolytic states were maintained throughout the streptokinase treatment period with thrombolytic activities, calculated from the radiochemical assays, of 100 to 500 μg. fibrin lysed per ml. plasma per hour. These activities far exceed any assayed in human subjects treated with adrenalin, electroshock, subjected to pyrogen injection or to procedures involving ischemia of an extremity (30) and are much greater than those developing post mortem after sudden death (31). Theoretically the plasma thrombolytic activities attained should be more than sufficient to produce rapid thrombolysis of emboli or thrombi contained within the lumen of vessels even if generous allowance is made for the poor blood flow adjacent to these regions. Clearly a theoretical contention of this nature requires the support of clinical trial and this trial, considered to provide practical demonstration of the contention's validity, is reported elsewhere (12).

Full activation of plasminogen contained in plasma would cause, were it not for the presence of plasma antiplasmin activity, rapid and extreme reduction of plasma components susceptible to its action and in fact it has been calculated that it would cause total destruction of fibrinogen contained in the body in 15 minutes (32). However, in practice, full activation of plasminogen causes relatively minor changes in plasma constituents susceptible to its action because of the high antiplasmin activity of plasma invariably found. The average normal plasminogen content of plasma is 3.5 casein units per ml. (calculated from Table I), but the average antiplasmin activity (calculated from the data of Table III) is 5.1 casein units per ml. As has been mentioned earlier, the calculation of antiplasmin values is to a degree arbitrary as it is influenced by the time allowed for plasmin antiplasmin interaction prior to completion of the assay, but our present data indicate that, at least in healthy persons, the antiplasmin content of plasma normally exceeds its potential plasmin activity.

The evidence advanced in this and preceding communications (30, 11) suggests certain new concepts concerning the nature of physiological and pathological fibrinolytic phenomena. The presence of plasminogen in plasma and the obvious manifestations of fibrinolytic phenomena in this fluid have focused attention on what are apparently associated consequences of the thrombolytic state rather than on the primary mechanism of thrombolysis. The hypothesis that best explains the present data would imply that plasma fibrinolytic phenomena (hereafter referred to as "fibrinolytic" phenomena) occur as an incidental consequence of the transport by plasma of plasminogen system components. Plasminogen is present almost exclusively as a plasma component, but physiologically (as concerns its function in thrombolysis) may be regarded as inactive in this milieu and its function appears to be to endow newly formed thrombi or other fibrinous deposit with a proenzyme, which on activation is capable of effecting its lysis. In the presence of plasminogen activators, by which thrombolysis may be mediated, the inhibitory action of plasma serves as a safeguard to prevent uncontrolled enzymatic

change in the plasma, but on occasion this safeguard proves insufficient and manifestations of "fibrinolysis" as a disease state become apparent.

Hemorrhagic phenomena are frequently observed in patients, who spontaneously develop "fibrinolysis" usually following obstetric complications or after surgery. However, owing to the relatively transient nature of the complication and the frequently critical condition of the patient, the opportunities for study of this state have been limited. The present investigation has provided a unique opportunity for such study as the 50 patients in our series experienced the biochemical changes incident to an intense thrombolytic ("fibrinolytic") state for continuous periods from five to 42 hours; in 34 of them the duration was 30 hours.

Experience shows that the hemorrhagic diathesis was not an invariable accompaniment of the thrombolytic state. Falls of plasma AcG occurred equally in those patients who did or did not exhibit the diathesis and appeared to exert an insignificant effect upon its pathogenesis. It is of incidental interest that the recently described compound, vitamin KS II (33), was shown in two patients to be ineffective in the prevention of the defect. A substantial rise of plasma antithrombin activity was invariably demonstrable preceding and during the course of the diathesis or its complications and its prevention by steroids markedly reduced the incidence of this diathesis. The one stage prothrombin time was closely correlated with the degree of plasma antithrombin activity (Figure 4) and unless the prothrombin time exceeded 30 seconds, gross evidence of the diathesis was lacking. However, bleeding occurred infrequently even in the presence of intense thrombolytic states and a severe coagulation defect. Several of our patients experienced both conditions for periods of many hours, the only clinical manifestations of the diathesis being related to bleeding from venipuncture sites with the subsequent formation of ecchymoses in these areas. These observations would seem to follow as a corollary to our findings that capillary fragility remained normal and that fibrin laid down in a plasminogen-poor or plasminogen-free milieu was virtually insusceptible to thrombolysis (11) because of lack of plasminogen within its substance. However the presence of a coagulation defect of sufficient degree could, but need not necessarily, impede hemostasis to such a degree that small hemorrhages secondary to the thrombolytic state might continue unchecked. von Kaulla and Swan (34) who investigated "fibrinolysis" secondary to the use of the heart-lung machine in open cardiac operations have noted similar findings and have come to the same conclusion.

Confirmatory to these general views is the evidence that Dicumarol®, which increased the magnitude of the coagulation defect, and local disease or trauma, which provided a focus for hemorrhage to occur, both acted as predisposing causes to the development of clinical hemorrhagic manifestations. Bleeding into the margins of wounds is a special case and occurs independent of the hemorrhagic diathesis, being a predictable result of the induced thrombolytic state and a manifestation of its activity. Nevertheless, if the wounds were in deep and inaccessible sites, we have regarded this feature as a total contraindication to the induction of the thrombolytic state. If anything less than the most meticulous hemostatic technique were employed at the original operation, the dangers of bleeding from a thrombosed, but unsutured vessel, must be considerable.

Plasma antithrombin activity developing during the thrombolytic state has properties different from the conventionally described entities antithrombins I-IV (35). There is, however, a close resemblance between the antithrombin activity found in our patients and that described recently by Loeliger and Hers (36) as "antithrombin V," which term they applied to the antithrombin activity apparent in the plasma of a patient suffering from rheumatoid arthritis. We have evidence to suggest that the antithrombin activity present in our patients was at least partly due to the presence of fibrinogen breakdown products but characterization is as yet insufficiently advanced to be certain that this factor represents the total antithrombin effect. However this finding might account, at least partly, for the dramatic effect of steroid administration in the prevention of the hemorrhagic diathesis. Following steroid administration, patients showed a greatly reduced degree of whole blood fibrinolysis over the control series ($p < 0.001$) and this change may have caused a diminished production of fibrinogen

breakdown products.[10] Also we have noted, though as yet this cannot be proven on a statistical basis, that severe manifestations of the hemorrhagic diathesis occurred only in those patients in whom relatively high free plasmin concentrations were demonstrable, a finding in accord with the suggestion that plasma antithrombin activity may be related to the presence of fibrinogen breakdown products.

Hitherto the term "fibrinolysis" or the "fibrinolytic state" has been used to embrace both thrombolytic and plasma proteolytic manifestations. The separation of this complex phenomenon into two distinct entities has important clinical and therapeutic implications. First, it is clear that thrombolysis may occur independently of plasma proteolysis and the independence of these two reactions was demonstrated in our patients. Secondly, hydrocortisone administration greatly reduced the incidence of undesirable proteolytic phenomena without impairment to the thrombolytic state; refinement of this and other precautions should permit the development of simple therapeutic regimes. Lastly, clarification of the mechanism and associated features of the enhanced proteolytic state has allowed of its recognition in a number of disease conditions (37) where its presence has not hitherto been described.

SUMMARY

1. Thrombolytic states of long duration (30 hours) and of great intensity (plasma activities of 100 to 500 μg. fibrin lysed per hour per ml. plasma) have been produced in man by the intravenous infusion of streptokinase.

2. Normally the thrombolytic state was well tolerated, but some patients developed a hemorrhagic diathesis of mild to moderate severity. The aberrations contributing to the development of this diathesis have been partially elucidated and the concurrent use of intravenous hydrocortisone has been shown to prevent or at least greatly reduce the severity of the hemorrhagic diathesis.

3. It was concluded that the induced thrombolytic state was well tolerated by man and that its use as a therapeutic tool in the treatment of thromboembolic disease was worthy of further investigation.

[10] The observation that fibrinogen levels in steroid treated patients did not differ significantly from those in the nonsteroid treated group does not exclude this hypothesis since fibrinogen breakdown products may influence the fibrinogen assay.

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THE MAINTENANCE OF A SUSTAINED THROMBOLYTIC STATE IN MAN. II. CLINICAL OBSERVATIONS ON PATIENTS WITH MYOCARDIAL INFARCTION AND OTHER THROMBOEMBOLIC DISORDERS * †

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Acute thromboembolic occlusive vascular disease is an important cause of human illness and the possibility of its treatment by measures designed to produce dissolution of the causative thrombus or embolus by enzymatic means has aroused much interest. Two preceding papers (3, 4) have been concerned with mechanisms of enzymatic clot dissolution and with the establishment of intense and prolonged thrombolytic states in man by the intravenous infusion of streptokinase.

These studies established the feasibility of using an induced thrombolytic state as a therapeutic tool for the treatment of thromboembolic disease in the human. However the effect of the thrombolytic state on infarcted tissue has never been tested and theoretically this effect could be harmful. Consequently the aim of the present clinical trial has been twofold, first to determine whether high levels of plasma thrombolytic activity were locally harmless to infarcted tissue and second to test the therapeutic value of this treatment. The present communication describes our experience with this type of therapy in several classes of human thromboembolic disease including patients suffering from early myocardial infarction.

The results suggest that high levels of plasma thrombolytic activity are harmless to infarcted tissue, including the myocardium, and that at least in a number of patients the clinical evidence was consistent with the view that thrombolysis of the causative thrombus or embolus had occurred.

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METHODS

Assay methods, materials and details of streptokinase [1] dosage were as previously described (3, 4). Biochemical and physiological findings obtained in the present series but pertaining to the induced thrombolytic state have been described elsewhere (4). All 45 patients described in this communication developed and sustained intense thrombolytic states (plasma activities of 100 to 500 μg. fibrin lysed per ml. plasma per hour) throughout the treatment period.

Myocardial infarction series. This group comprised 22 patients of whom five were female. The average age of the group was 58 years and the age distribution: 30 to 40 years, one patient; 40 to 50 years, five patients; 50 to 60 years, eight patients; 60 to 70 years, two patients; and 70 to 80 years, six patients.

No patient was included in the series unless the clinical history and findings were of diagnostic significance, the electrocardiographic changes were confirmatory and review of serial transaminase (SGO-T) assays sustained the diagnosis. Theoretically greater benefit should follow the early rather than the late use of thrombolytic therapy and consequently patients were treated as soon after the onset of infarction as was practicable. The patients have been divided into two groups, a main one comprising 19 patients in whom thrombolytic treatment was started five to 16 hours (average nine hours) from the time of onset of symptoms and a subsidiary one of three patients in whom treatment was delayed 34 to 65 hours after the onset of the illness. All of the latter patients died and their course is considered in the section on autopsy evidence.

The main group contained five patients known to have been hypertensive for three to 15 years, two of whom had radiologically demonstrable cardiac enlargement. Two patients were known to have had previous attacks of myocardial infarction and this diagnosis had been strongly suspected in a third. Three patients were obese (20 to 40 per cent over ideal weight) and four patients were considered to have suffered from unusually severe and protracted pain following the onset of infarction. Though three patients showed substantially depressed blood pressure, signs of shock were present in only one. Two pa-

[1] Streptokinase was kindly supplied by Dr. J. Ruegsegger, Lederle Laboratories, Pearl River, N. Y.

tients showed signs of early congestive failure but these were mild. All patients received streptokinase by intravenous drip for 30 hours in sufficient doses to produce a sustained and intense thrombolytic state and the last eight patients in the series received supplemental intravenous hydrocortisone for the duration of the infusion period (4). The initial organization of the trial involved a "double-blind" design and the decision as to treatment in the first 23 patients was made by the drawing of a lot. However this design was found to impede the collection of physiological data (4) and its use was abandoned, but later reference is made to the 11 control patients in the series.

RESULTS

Pulmonary embolism, thrombophlebitis, thrombotic arterial occlusion, and coronary insufficiency

Case histories 1 through 23 (see Appendix) describe the effects of the induced thrombolytic state in these categories of patients.

Acute myocardial infarction

The clinical course of the patients during the 30 hour streptokinase treatment period was relatively uneventful and there were no deaths at this time. Eight of the patients, initially considered to be gravely ill, improved considerably as did those others whose conditions appeared to be less serious. Two patients after six hours' treatment experienced a recurrence of moderately severe chest pain of one to two hours' duration; in both instances the blood pressure fell and remained unstable for eight and 12 hours, respectively, though other signs of shock were absent. One patient experienced moderate chest pain after 12 hours' treatment but the blood pressure remained stable and the pain only lasted 30 minutes. Two of the hypertensive patients showed a fall of blood pressure to normotensive levels, a fall that was maintained for the remainder of the hospital course. Two patients, whose blood pressure had been initially depressed, showed a rapid rise while on streptokinase treatment. All patients were started on Dicumarol® therapy prior to the end of the infusion and, except in two instances, were maintained on this drug for the remainder of the hospital course. Nine patients received anticoagulant treatment after discharge from the hospital.

Later course

Following the initial treatment phase, there was one death in a 72 year old male, who died three weeks later from recurrent infarction; the autopsy findings are described later. Three patients, all males, experienced episodes of chest pain that may have been indicative of recurrent infarction three, six and 10 days, respectively, after the onset of the original infarction. The pain in each instance was of less than two hours' duration, but in two of the patients, transient electrocardiographic changes occurred, though in all of them serial transaminase assays remained normal and no other evidence of deterioration was obtained. Four other patients complained intermittently throughout their hospital course of mild precordial aching sensations. However, two were nervous patients and in the case of an obese 74 year old woman abdominal discomfort may have contributed to her difficulty. The convalescence of a 53 year old male was delayed by the development, three weeks after admission, of a severe attack of benign pericarditis, which ultimately resolved without disability. Embolic complications were absent from this series. Following discharge from hospital, two patients, respectively six and 12 weeks later, experienced bouts of chest pain associated with the development of minor electrocardiographic abnormalities; in each instance the pain rapidly disappeared, serial transaminase assays remained in the normal range and the patients are now symptom free.

Followup

Followup was usually conducted six months later but in four instances only the two month followup is available, though the patients are known to be alive and well after five months. In two instances followup was oral but all the other patients were fully investigated. Eleven patients were symptom free and pursuing their normal vocation or activities (though in the case of four elderly patients these were limited); four patients, two of whom were working, complained of dyspnea on unusual exertion though their normal activities were not limited by this disability. The remaining three patients, two of whom were working, experienced occasional very mild chest discomfort on moderate exertion. Physical examination revealed no unsuspected findings and

clinically we gained the impression that the group had made a satisfactory recovery. Radiological examination disclosed no evidence of aneurysm formation and in 13 of the patients the size of the heart shadow was within the limits of normal. Four patients showed mild to moderate cardiac enlargement, in all cases with enlargement of the left ventricle, but in two of them, previous chest films showed that this enlargement had been present prior to the present illness. The other two patients, in whom previous chest films were not available, had both previously suffered from myocardial infarction and in one of the patients, a clinical diagnosis of cardiac enlargement had been made prior to the present illness.

Electrocardiographic evidence

Serial electrocardiograms were recorded several times on the first and second day of therapy and subsequent records were then taken daily until the clinical course of the patient had stabilized. In all cases, the electrocardiograms were considered to be either diagnostic of acute myocardial infarction or consistent with the diagnosis. Weekly or semiweekly records were then obtained until discharge from the hospital. In most instances the electrocardiographic evolution of the infarct was not remarkable. However, in three cases, a subsequent bout of chest pain during the period of convalescence was accompanied by a recurrence of ST segment elevations in the leads previously involved in the original infarct, but in none did the Q waves change and the SGO-T determinations remained within normal limits.

The electrocardiograms were read as showing anterior or anteroseptal myocardial infarction in 15 cases, diaphragmatic (posterior) in six cases and left bundle branch block was present in one. Significant Q waves were present initially or developed subsequently in 20 cases. Of the 18 surviving patients, followup records (usually five to six months after the acute infarct) were obtained in 14 instances and in the remaining four patients, the followup was considered to be of insufficient length to permit adequate assessment. Four of the patients showed a normal electrocardiogram on followup examination (one of the four has a slightly prolonged P-R interval which was probably present prior to the infarct). Three of the

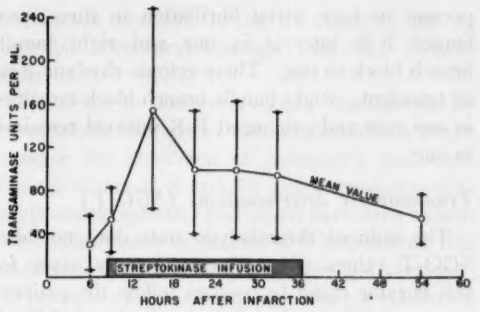

FIG. 1. SERIAL SGO-TRANSAMINASE VALUES IN MYOCARDIAL INFARCTION (SK TREATED)

The average delay between the time of infarction and the start of streptokinase treatment was nine hours (range five to 16 hours). The highest average SGO-T values were recorded in the specimens drawn just after the start of treatment (12 to 18 hour period) and the values thereafter declined.

four patients with normal followup records had developed significant Q waves during the acute infarction. Of the 10 patients with abnormal followup electrocardiograms three were known to have had abnormal records prior to the acute infarction. In the remaining seven patients, previous electrocardiograms were not available.

Arrhythmias or conduction disturbances developed in seven patients. In six of these patients arrhythmias or conduction disturbances were present prior to treatment with streptokinase, although other abnormalities in rhythm or conduction subsequently occurred in several of these patients during or following streptokinase therapy. In one of the seven patients, ventricular premature contractions, auricular fibrillation and prolonged intraventricular conduction occurred after the start of therapy, whereas no arrhythmia or conduction disturbance was previously recorded. The conduction disturbance in this case was thought to be due to quinidine therapy and the patient subsequently made an uneventful recovery. In one of the patients who died, right bundle branch block, ventricular premature contractions and atrial tachycardia with block were present intermittently before death. In another, ventricular premature contractions and intraventricular conduction disturbance were present and this patient also died. In the remaining five cases who survived, ventricular premature contractions were

present in four, atrial fibrillation in three, prolonged P-R interval in one and right bundle branch block in one. These ectopic rhythms were all transient. Right bundle branch block remained in one case and prolonged P-R interval remained in one.

Transaminase determinations (SGO-T)

The induced thrombolytic state does not alter SGO-T values (4) and consequently assay for this enzyme could be used to follow the patient's progress. Figure 1 shows the combined SGO-T determinations in Group 1 patients over a two day period; the time scale corresponds, in the conventional manner, to hours after the onset of infarction. The shape of the curve appears to differ from that usually found in myocardial infarction as peak values were noted as early as 15 hours and the average level had almost returned to normal by the third day. Comparison between the streptokinase treated patients and those in the double-blind control group would at first sight appear to support this hypothesis, for though transaminase values in the two groups were initially similar ($p > 0.1$), the later readings (obtained between 30 to 50 hours) showed the control values to be higher ($p = 0.05$). However, comparatively little weight can be attached to this finding as the control data show evidence of sampling bias.

Urokinase excretion

An interesting and unexplained finding in the myocardial infarction patients concerned their urokinase excretion rates (rates of urinary plasminogen activator excretion). Normally patients suffering from myocardial infarction show a substantial rise in urokinase excretion rates for the month following its onset ($p < 0.001$) (5), but in the streptokinase treated group this rise occurred faster and was of greater magnitude ($0.01 > p > 0.001$) than in the control group. This finding may be of importance (5), but, at present, there is insufficient collateral data for its proper interpretation.

Autopsy evidence from the myocardial infarction series

Four patients died, though in the case of a 74 year old woman, originally in shock and in whom only three hours' treatment was given, autopsy permission was refused.

A 72 year old male died suddenly three weeks after his initial treatment while receiving Dicumarol®. Autopsy revealed a large normally healing infarct (estimated to be two to four weeks old) and in its center a small very fresh infarct related to a new vessel thrombus. Though the coronary vessels were grossly arteriosclerotic, search revealed no older thrombus to correspond with the original infarct. A 53 year old male, admitted 48 hours after the onset of infarction, showed shock, paroxysmal auricular fibrillation and an SGO-T of over 200. He died 14 hours later, probably from ventricular fibrillation. Though autopsy revealed a huge posterolateral infarct with septal involvement, careful dissection of the coronary vessels revealed only the presence of mild coronary sclerosis and failed to demonstrate the presence of a coronary thrombus. A 49 year old male, admitted to hospital 34 hours after the onset of infarction, was in gross congestive failure and his SGO-T peaked at 340. Though his congestive failure showed an initial response to treatment, he died from this cause, one day after the termination of streptokinase therapy. Autopsy revealed an old large posterior wall infarct and a new extensive anterolateral area of infarction. A small thrombus was found arising from an arteriosclerotic plaque in the left main coronary artery, but since its presence was only demonstrable by microscopy and it appeared to be very fresh, it may have formed after the end of the streptokinase treatment period. Neither in these nor in other patients (4) were myocardial lesions other than those attributable to the primary disease state observed.

Clinical toxicity

Neither pyrexia nor hypotension was noted after streptokinase administration. The sole serious or potentially serious toxic effects of the treatment have lain in the development of a hemorrhagic diathesis, secondary to the induced thrombolytic state (4). This complication can, at least in part, be prevented by steroid administration (4). One patient developed, 24 hours after the termination of the infusion, a 5×4 cm. erythematous area around the infusion site: This lesion,

thought to represent a sensitization reaction, disappeared three days later. Though streptokinase solution occasionally has become infiltrated at the infusion site, reactions have not resulted except in one patient in whom massive infiltration of the leg was produced and in this case the reaction was slight. Minor thrombophlebitis subsequently developed at the site of the indwelling needle in four patients.

DISCUSSION

The evaluation of a new therapeutic procedure in disease states that exhibit variable morbidity and mortality patterns is fraught with the possibility of error. Evaluation must necessarily rest on a quasistatistical basis involving the observation of a number of favorable results where the contrary would normally be expected. Obviously, whatever the outcome in an individual case, chance can never be excluded. Accordingly the total case material has been presented, individually described where the usual outcome would be customarily judged on the severity of the presenting disease state (see Appendix), but grouped together, as in the myocardial infarction patients, where the initial findings constituted a less certain guide to the eventual outcome.

The case histories 1 through 23 (described in Appendix), comprising patients suffering from disease states other than myocardial infarction, are consistent with the view that, at least in a number of patients, *in vivo* thrombolysis was produced as a result of the treatment. The series of events that occurred in Patients 1 through 6, suffering from pulmonary embolism, in Patients 7 through 10 suffering from thrombophlebitis of the large veins, in Patients 18 and 19 suffering from arterial obstruction and Patient 23 with a fibrinous exudate in the anterior chamber of the eye, though perhaps individually explainable as a series of chance findings, are, taken as a whole, difficult to reconcile with such a view. A corollary to these findings would indicate that restoration of blood flow to an infarcted area is not only of over-all benefit but also without deleterious effect. Where failure was met with Patients 11 through 14 suffering from thrombophlebitis and Patients 15 through 17 suffering from arterial disease, there appeared to be an adequate reason for this result, either on the grounds of the unsuitable nature of the disease condition or on the basis of the irreparable structural damage that had already occurred.

Theoretically there is a danger that the use of thrombolytic therapy in venous thrombosis might increase the likelihood of pulmonary embolism. Though Cases 2, 8 and 10 experienced transient symptomatic episodes that could have been indicative of pulmonary embolism, it is emphasized that confirmation of this diagnosis was not obtained; the patients were suffering from disease conditions where spontaneous pulmonary embolism is common and unusual attention was being given to the patient's symptomatic complaints. We do not regard the danger of pulmonary embolism as a contraindication to the treatment, since even if it were to occur, rapid lysis of the embolus would be expected to take place (see Patients 1 through 6).

It is our present view, based solely on theoretical conjecture, that thrombolytic treatment should be followed by anticoagulant treatment. However, not all our patients have been given anticoagulant treatment and so far we have seen no ill effects from its omission.

Myocardial infarction presents a difficult therapeutic problem, because of the numerous etiological factors involved in its causation. The rapid dissolution of a coronary thrombus by enzymatic means could result in reduction of the final area of muscle infarction, reduction of the degree of electrical instability present during the early critical phase of infarction and in the prevention or removal of mural thrombi. However, the thrombolytic state could conceivably cause deleterious changes within the infarcted area and perhaps increase the tendency to or the degree of subintimal hemorrhage. Moreover in those instances of infarction, unrelated to thrombosis, benefit could only occur by the prevention of or by the lysis of mural thrombi. These problems were similar in substance but more acute in nature than those involving the therapeutic use of anticoagulants in this condition. In contrast, while the aim of anticoagulant treatment is to limit myocardial damage and prevent further spread of infarction, the aim with thrombolytic therapy is to restore the circulation and permit the restoration of function to still viable, but otherwise doomed, tissue. Clearly the timing of thrombolytic therapy is criti-

cal and, theoretically at least, it should be started as soon as possible after the onset of infarction. This therapeutic indication is fortunate since by far the greatest mortality occurs in the first 24 hours of the disease and conventional therapy is largely unavailing. Furthermore, thrombolytic therapy if used late in the disease, after the formation of mural thrombi, might carry a serious risk of causing arterial embolism.

Evidence concerning the effect of the thrombolytic state on the myocardial lesion was of a dual nature, that derived from the living and that obtained by postmortem examination. The three patients treated late in the illness, 34 to 65 hours after its onset, died during the acute phase of the disease. These patients were not included in the main group as the delay placed them outside the scope of the study and their treatment was only reluctantly undertaken as a measure of last resort. Postmortem examination was also made on the one patient in the main series who died three weeks after his initial treatment. Pathological study was consistent with the belief that *in vivo* thrombolysis had been produced and also with the view that the thrombolytic state had exerted no harmful effect upon the evolution of the infarcts. Of collateral interest are two reports (6, 7) offering suggestive evidence that the relief, by enzymatic means, of acute coronary obstruction in the dog lessens the final degree of myocardial damage.

Patients treated early in the course of the disease all survived the acute phase of the illness, though one of the 19 patients died three weeks later from recurrent infarction. It is probable that these patients suffered from myocardial lesions of greater than average severity since the incidence of features indicative of a poor prognosis was high and the referring physicians naturally tended to favor the use of this treatment in patients whose prognostic outlook, under conventional treatment, was deemed to be relatively unfavorable. The hospital course and the followup period have been devoid of unusual incident and it is our impression that the survivors have made good recoveries. Radiological examination has failed to reveal aneurysm formation and the incidence of cardiac enlargement has been very low. The fact that four of 14 patients showed normal electrocardiographic findings six months after the attack is unusual, especially when three of these 14 patients were known to have shown abnormal ECG findings prior to the attack. Though the mortality rate of the group appeared to be unusually low testing of its statistical significance against the mortality rate in patients treated as double-blind controls (four out of 11 died) and also against the hospital mortality during the time of the trial (19.5 per cent) gave in each instance $p = 0.2$ (χ^2 with Yates correction). Thus though our findings may suggest that the patients derived clinical benefit from the treatment, on the crucial question of mortality we would have seen as favorable a series, by chance alone, one trial in five. Clearly, while the number of patients treated has been insufficient to arrive at a decision that benefit occurred, it does appear to be sufficient to give considerable assurance that certain theoretical objections that could be raised to this form of treatment are without practical basis.

Though clinically we have been gratified by the results of this treatment we would emphasize that these observations are of a preliminary nature and serve only to establish that the present therapeutic approach is feasible. Clinical, pathological and biochemical evidence all indicate that plasma thrombolytic states are harmless to the infarcted myocardium and that the development of thrombolytic therapy for the treatment of myocardial infarction will not be impeded by deleterious consequences, secondary to its primary effect.

APPENDIX

Case histories 1 through 23. The effect of the induced thrombolytic state upon the clinical course of patients suffering from pulmonary embolism, thrombophlebitis, thrombotic arterial occlusion and coronary insufficiency.

Pulmonary embolism

Six patients, all gravely ill, have been treated. However, absolute proof of the diagnosis was lacking in *Case 1,* a 32 year old male. Though there was adequate clinical evidence to support the diagnosis, his poor general condition precluded the obtaining of radiological confirmation. He died two days after streptokinase treatment from congestive cardiac failure of rheumatic etiology and, at autopsy, dissection of the pulmonary vascular tree disclosed no abnormality.

Case 2. The successful treatment of a 53 year old farmer, suffering from acute cor pulmonale, secondary to repeated pulmonary emboli has been previously reported (1).

Case 3. A 38 year old female gave a history suggestive of four episodes of pulmonary embolization in two days; physical signs and chest film confirmed the diagnosis. Her last episode of pulmonary infarction, which occurred two hours before the start of treatment, was massive and had caused brisk hemoptysis. Rapid clinical improvement followed streptokinase therapy, no further emboli occurred and signs of phlebothrombosis present in the calf rapidly regressed. Radiological examination showed almost complete clearing of the chest in a two day period. This patient was awaiting operation for uterine myomata and it was noteworthy that successful treatment was impeded neither by the presence of vaginal bleeding nor by the hemoptysis. The extent of bleeding was not increased from either site by the establishment of a thrombolytic state.

Case 4. A 54 year old male had been treated one year previously for multiple pulmonary embolization secondary to thrombophlebitis. Conservative management led to an excellent functional recovery. Three days prior to admission he experienced pain and tenderness in the left popliteal region and shortly prior to admission, left-sided pleuritic chest pain, cough, sweating and dyspnea. He was acutely ill exhibiting cyanosis, tachycardia, tachypnea and had a cold clammy skin with a blood pressure of 80/60. The electrocardiogram showed a partial right bundle branch block and other signs suggestive of pulmonary embolism. Twelve hours treatment with heparin and oxygen with a continuous norephinephrine drip to maintain the blood pressure produced no improvement. Rapid improvement followed streptokinase treatment and after 10 hours his blood pressure rose to normal values and remained stable without the use of norepinephrine. Cyanosis disappeared and oxygen was dispensed with at the end of streptokinase treatment. His transaminase (S-GOT) rose to over 200 units per ml. and, though this finding was regarded as rather unusual for pulmonary embolism, his electrocardiogram returned to normal after five days. Three days later despite the use of Dicumarol®, the physical signs of acute thrombophlebitis were apparent in the left thigh. In view of his history, left femoral ligation was performed and he was discharged shortly afterwards symptom free except for the residual functional effects of the femoral ligation.

Case 5. A 47 year old male was discharged from hospital 14 days after the performance of a subtotal gastrectomy. Three days later the patient was seized by shaking chills and noted the sudden onset of severe pain in the right chest. He was cyanosed, hypotensive and had physical findings consistent with multiple pulmonary embolism, a diagnosis confirmed radiologically. The patient's condition deteriorated rapidly and he died 16 hours later, but for the last seven hours he received streptokinase therapy. Autospy revealed the primary cause of death to be generalized peritonitis secondary to a leaking suture line, but though three areas of recently infarcted lung tissue were found, dissection of the pulmonary vascular tree revealed no emboli.

Case 6. A 43 year old female developed, following hysterectomy, pelvic vein thrombosis. Despite adequate treatment with Dicumarol®, repeated pulmonary embolism occurred over a 35 day period. Following streptokinase therapy, her chest film cleared and no further episodes of embolization occurred.

Thrombophlebitis

Eight patients were treated in this group and though patients suffering from severe clinical manifestations secondary to lesions of large veins responded well to streptokinase treatment, other patients with lesions of small veins and few clinical manifestations, except those of local disease, apparently did little or no better than with conventional therapy. We attribute this somewhat paradoxical response to the fact that whereas with disease of the large veins clinical disability is related primarily to venous obstruction, this fact is of little importance in disease of small veins, inflammation of the vein wall being the predominant clinical lesion.

Case 7. A 44 year old woman, who had had several hospital admissions on account of idiopathic steatorrhea, developed acute ileofemoral thrombophlebitis secondary to an indwelling catheter in the inferior vena cava. Three days' streptokinase therapy produced a rapid resolution of the condition. Her hospital stay was prolonged on account of the intestinal disorder but on discharge there was no evidence of venous insufficiency.

Case 8. A 53 year old male suffered from ileofemoral thrombophlebitis secondary to trauma and responded rapidly to streptokinase therapy. During the course of treatment he developed transient symptoms suggestive of pulmonary embolism but further evidence to support this diagnosis could not be obtained. On discharge from the hospital eight days later there were no signs of venous insufficiency.

Case 9. A 78 year old man with gouty arthritis developed ileofemoral thrombophlebitis. The response to treatment was slower than in the other two cases and he also developed an exacerbation of gout. However his leg was free from edema on discharge though he was wearing an elastic support. Two months later ileofemoral thrombophlebitis recurred, temporary shortage of streptokinase prevented his retreatment and despite the use of anticoagulants he developed a marked post-phlebitic syndrome.

Case 10. A 46 year old male developed calf, popliteal and femoral thrombophlebitis, following injury. Response to streptokinase therapy was very satisfactory and he left the hospital without disability. During the first day of treatment he experienced transient symptoms that could have been due to pulmonary embolism but investigation failed to confirm the diagnosis.

Cases 11 and 12. Both suffered from long standing thrombophlebitis of the calf and though both were improved following streptokinase therapy, the improvement could well have been a chance finding and was unimpressive.

Cases 13 and 14. These two patients had thrombophlebitis of superficial veins and though clinical symptoms abated rapidly following treatment, venous cords remained palpable.

Peripheral arterial occlusions

Six patients have been treated.

Cases 15, 16 and 17. All were elderly patients suffering from acute obstruction to the femoral artery. The patients were treated with streptokinase after extensive and wholly unsuccessful surgical intervention had been undertaken in each patient. Though in one instance a previously absent popliteal pulse returned after treatment, lasting benefit was not obtained. Two patients died, one from myocardial infarction six weeks later, the other from acute renal tubular necrosis following surgery. Probably the condition of the artery wall after unsuccessful surgical intervention is such as to preclude other treatment.

Case 18. One year previously an arterial homograft had been inserted to bridge the left iliac and femoral arteries in this 61 year old female. The limb circulation failed suddenly and, on examination, signs of acute arterial insufficiency were apparent midway up the thigh. The graft, which was easily palpable throughout the greater part of its course, was hard and pulseless. Eight hours after the start of streptokinase therapy the superior one-third of the graft was pulsatile. However, by next morning when treatment was restarted, the whole graft was again hard and pulseless and further treatment failed to change its condition. Nevertheless, perhaps because the graft thrombosis was an incidental finding, the circulation of the leg improved during therapy. Gangrene did not develop and the patient's ambulatory ability on discharge from hospital was similar to that present before her current admission.

Case 19. A 66 year old female, bedridden as the result of a previous vascular accident, was admitted suffering from pneumonia of great severity. Despite the exhibition of antibiotics her condition remained serious. Blood pressure could only be maintained by the administration of intravenous norepinephrine over the first three days. On the fourth day, two localized patches of gangrene were apparent on the left limb in the distribution of the anterior tibial artery. On the fifth day she developed signs of acute arterial insufficiency in the limb with the border of temperature change 5 cm. above the knee. During streptokinase therapy oscillometric pulsations were again recorded in the lower limb, muscle power returned to the foot and the temperature differential between the legs diminished to an insignificant difference. However the original patches of gangrene were unaltered by the treatment and since owing to her previous bedfast state mobilization was impossible, below the knee amputation was performed.

Miscellaneous

This group consisted of three patients with coronary insufficiency and one patient with an intraocular fibrinous exudate.

Case 20. A 61 year old male suffering from mild coronary insufficiency received therapy; his course thereafter was uneventful.

Case 21. A 44 year old male experienced an attack of severe and prolonged chest pain with electrocardiographic abnormalities and a slightly raised SGO-T assay; however Q waves did not subsequently develop. Following 30 hours' streptokinase treatment his course was satisfactory and he is now, five months later, symptom free.

Case 22. A 64 year old emaciated female was admitted with gross congestive cardiac failure, an enlarged heart and severe chest pain. After an initial response to therapy she suddenly died three weeks later. Autopsy revealed severe coronary artery disease, several areas of old healed infarcted tissue and one fresh infarct estimated to be no more than two days old.

Case 23 (1). A 71 year old male developed an intraocular infection following a cataract operation. Despite intensive antibiotic therapy a dense fibrinous exudate developed and completely filled the anterior chamber. Four days of streptokinase treatment was given and serial slit lamp examination revealed progressive resolution until, at the end of treatment, the anterior chamber was free of exudate. It is known that spontaneous resolution of such exudate seldom if ever occurs and this patient was thought to provide a rather unique demonstration of *in vivo* fibrinolysis.

SUMMARY

Fifty patients suffering from several varieties of acute occlusive thromboembolic disease, including 22 patients suffering from early acute myocardial infarction, were treated by the intravenous infusion of streptokinase in massive doses and for prolonged periods. Intense plasma thrombolytic activity (100 to 500 μg. fibrin lysed per hour per ml. plasma) developed and was maintained for 30 or more hours.

The thrombolytic state produced no important deleterious consequence and there was evidence, at least in a number of patients, to suggest that *in vivo* thrombolysis had been produced. The low mortality rate and the relative absence of complications experienced by the myocardial infarction patients suggested that the induced thrombolytic state was harmless to the infarcted myocardium.

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THE DIFFERENTIAL RESPONSE OF THE ADRENAL CORTEX AND MEDULLA TO BACTERIAL ENDOTOXIN *

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The administration of bacterial endotoxin to an animal results in a characteristic series of events, including hypotension, hyperglycemia, changes in the white cells and, at high dosage levels, death (1). The mechanism by which endotoxin produces these effects has not been defined and search for a common mode of action has not yet been fruitful. However, it has been noted on several occasions that there are many similarities between the systemic effects of endotoxin and epinephrine. It has therefore been speculated that some, if not most, of the effects of endotoxin are due to a liberation of epinephrine resulting from endotoxin stimulation of the adrenal medulla.

Since there is little evidence available to prove or disprove this concept, the present studies were set up to determine the effect of bacterial endotoxin on adrenal medullary function. It was thought desirable to use a technique whereby samples of adrenal venous blood could be obtained and analyzed for catechol amines noting the response to various dosages of endotoxin. This would provide a more direct measurement of adrenal medullary function than the determination of peripheral blood levels of the medullary hormones. In addition to determining the adrenal venous output per minute of catechol amines, measurements of the output of 17-hydroxycorticosteroids were made in order to contrast adrenal cortical and medullary responses. Similar experiments were performed in animals with spinal cord transections to provide information concerning the pathways by which adrenal cortical and medullary activation took place.

MATERIAL AND METHODS

Healthy adult mongrel dogs ranging in weight from 10 to 18 Kg. were used in these experiments. Cannulations of the lumbo-adrenal vein were carried out according to the technique of Hume and Nelson (2). This technique allows intermittent collection of adrenal venous blood to be made in the unanesthetized, unstimulated animal. Spinal cord transection was performed in four animals through a dorsal midline incision. Transection of the spinal cord between C-7 and T-1 was accomplished with a wire loop. Blood pressures were maintained in the immediate post-transection period by the intramuscular injection of Neo-Synephrine®. Spinal cord sectioned animals were maintained in the postoperative period by the intravenous administration of 500 ml. of 5 per cent dextrose in saline on each of the first two postoperative days, followed by food and water *ad libitum* after that. The animals were supported in a sling during portions of the day and this allowed them to take food and water without assistance. Antibiotics consisting of penicillin, 200,000 units per day, and streptomycin, 200 mg. per day, were given intramuscularly and the animals were turned frequently in the first few days following surgery. With adequate attention to details it was possible to keep cord sectioned animals in good condition with little loss in weight and vigor.

The day after adrenal vein cannulation, dogs were placed on a laboratory table and control samples of adrenal venous blood were taken. Rectal temperatures were obtained throughout the course of the experiments by means of thermistors inserted at least three inches into the rectum. It has previously been found that Nembutal® markedly depresses the secretion of epinephrine in response to a variety of stimuli and for this reason animals were unanesthetized. Careful selection of animals for these experiments was necessary so that they would not be excitable and they were conditioned to the presence of laboratory workers and the attention of laboratory personnel coincident with obtaining frequent samples of adrenal venous blood. Usually the head of the dog was covered with a sheet to reduce apprehension as a result of environmental activity and an attendant stayed with the dog to pet him if signs of restlessness occurred. In the first few minutes of sample collections, occasional high values for catechol amines were observed, but most animals returned to basal levels and stayed there for long periods if unstimulated.

Purified endotoxin of the Boivin type derived from *E. coli* was prepared by the method of Spink and Anderson (3).[1] Endotoxin solutions were made up so as to contain 0.1 mg. per ml. of saline and all endotoxin injections were given intravenously in a period of approxi-

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† Markle Scholar in Medical Sciences.

[1] Obtained through the courtesy of W. W. Spink and J. C. Melby.

TABLE I

Output per minute of E–NE from right adrenal gland after endotoxin administration *

Experiment no.	Dose endotoxin	Control values	Minutes†	Adrenal venous catechol amines (μg./min.)								
				10	20	30	40	50	60	90	120	150
	mg.											
1	0.5	0.02		0.14	0.18	0.56	0.84		1.22	1.05	1.24	0.46
2	0.2	0.05			0.57		0.40		0.32	0.06		0.02
3	0.2	0.19		0.18		0.56	0.47		0.16		0.06	
4	0.2	0.19			0.37	0.30	0.41	0.36		0.78		
5	0.2	0.07		0.13	0.50	0.67	0.65		0.34	0.12		
6	0.2	0.04		0.06	0.11	0.77		2.66				
7	0.01	0.03		0.05		0.15	0.14		0.18		0.17	
8	0.01	0.04		0.06		0.43		0.23		0.14	0.08	10
9	0.01	0.14		0.15		1.71	1.91		1.86	1.08	0.94	0.40
10	0.01	0.00		0.01		0.01		0.05	0.07	0.07	0.10	
11	0.01	0.03		0.06	0.04	0.04		0.01				

* All animals developed from 1 to 3° C. fever within two hours after endotoxin.
† Time after endotoxin administration.

mately 10 seconds. In general, two schedules of dosages were used, one of 0.01 mg., the other of 0.2 mg. It had previously been found that 0.01 mg. elicited an adrenal cortical response, fever and hypotension without death and 0.2 mg. represented the maximal tolerated dose without a fatality rate of over 10 per cent in normal dogs.

Epinephrine and norepinephrine determinations were carried out by a modification (4) of the method of Weil-Malherbe and Bone (5). Values are expressed in the results as total catechol amines (E-NE) although separate epinephrine and norepinephrine determinations were performed. This is because dihydroxyphenylethylamine and dihydroxyphenylalanine form fluorescent products with the methods used and therefore it is most accurate to report changes in total catechol amines. However, most of the increase in catechol amine output that took place was due to the epinephrine component, as measured. 17-Hydroxycorticosteroid determinations were performed according to the method of Nelson and Samuels (6). In some of the experiments, after one to two hours a dose of 25 units of corticotropin was given intravenously, following which more samples were obtained. Adrenal cannulation was carried out from one to 21 days after cord section and in all experiments endotoxin was administered on the day after adrenal vein cannulation. Blood pressure determinations were carried out in some experiments with an arterial polyethylene catheter connected to a mercury manometer.

RESULTS

A preliminary set of experiments was carried out in which only adrenal venous epinephrine and norepinephrine outputs (E-NE) were obtained following endotoxin administration. Reference to Table I indicates the pattern of response in these animals. Eleven dogs were given endotoxin at a dosage range from 0.01 mg. to 0.5 mg. All these animals manifested a fever of greater than 1.0° C. two hours after endotoxin administration. There was no correlation between the degree of body temperature elevation and dosage of endotoxin administered. In Experiment 1, the animal was given 0.5 mg. of endotoxin and demonstrated a significant E-NE response at 30 minutes, followed by increasing secretion up to one hour after endotoxin administration and then return towards normal after two and one-half hours.

Five animals given 0.2 mg. of endotoxin responded with a significant increase of E-NE output, with maximal levels 20 to 30 minutes after drug administration. Two of five dogs given 0.01 mg. of endotoxin had a significant adrenal medullary response, with Dog No. 9 attaining levels as high as 1.91 μg. per minute 40 minutes after endotoxin. Corticotropin given during declining E-NE levels did not stimulate the adrenal medulla.

A second set of experiments was then carried out in which 17-hydroxycorticosteroids were determined in addition to the catechol amines and these results are shown in Table II.

Dosages in this group of experiments range from 0.01 mg. to 0.5 mg. and all animals demonstrated fevers of over 1.0° C. In addition, all animals manifested maximal adrenal cortical stimulation as demonstrated by failure to increase corticosteroid output by corticotropin administration. In Experiment 1A, at a dosage of 0.01 mg. of endotoxin, when maximal adrenal cortical stimulation and fever occurred, there was no increase of catechol amine secretion. Similar results were ob-

TABLE II

Output per minute of E–NE and 17-OH from right adrenal gland after endotoxin administration *

Experiment no.	Substance measured	Dose endotoxin	Control values	Minutes†	Adrenal venous output (μg./min.) 0–20	20–40	40–60	60–90	90–120
		mg.							
1A	17-OH	0.01	1.2		1.3	19.4	23.6	29.4	
	E–NE		0.24		0.12	0.18	0.18	0.13	
2A	17-OH	0.01	1.8		7.0	19.6	19.2		
	E–NE		0.12		0.05	0.09	0.10		
3A	17-OH	0.01	1.6		0.8	14.0	36.0	29.8	16.2
	E–NE		0.10		0.17	0.10	0.19	0.21	0.10
4A	17-OH	0.50	2.6		11.6	13.4	11.8	12.6	10.6
	E–NE		0.25		0.15	3.32	1.35	1.28	0.89
5A	17-OH	0.10	7.8		3.8	23.2	19.4	19.6	20.4
	E–NE		0.05		0.06	0.20	1.09	1.05	0.94
6A	17-OH	0.10	0.6		4.0	14.4	26.0	27.2	
	E–NE		0.02		0.05	0.06	0.34	0.20	
7A	17-OH	0.01	1.4		4.4	22.6	29.0	19.6	23.2
	E–NE		0.02		0.03	0.06	0.12	0.18	0.18

* In several of the experiments, the administration of 25 units of ACTH had no effect on E–NE levels and did not cause a further increase in 17-OH after endotoxin. All animals developed fever of 1 to 3° C. after endotoxin.
† Time after endotoxin administration.

tained in Experiments 2A, 3A and 7A all of which involved the intravenous administration of 0.01 mg. of endotoxin. These experiments demonstrate clearly that a dose of endotoxin which uniformly results in the production of fever and maximal adrenal cortical stimulation may fail to result in adrenal medullary stimulation in addition. Experiments 4A, 5A and 6A using doses of 0.1 mg. or more led to significant E–NE output increases. Throughout this set of experiments exogenous corticotropin did not further increase the output of 17-hydroxycorticosteroids which followed endotoxin stimulation.

Reference to Table III indicates the results of experiments in which dogs with spinal cord section and adrenal cannulae were given small and large doses of endotoxin. All animals with spinal cord transection responded with the expected maximal output of 17 hydroxycorticosteroids and with a fever of over 1.0° C. at one and one-half hours following endotoxin administration. In contrast to the fever and adrenal cortical responses, none of the dogs responded to 0.01 mg. of endotoxin with an increase in E–NE and another injection at 90 minutes of 0.2 mg. failed to alter adrenal medullary output of catechol amines from basal levels. Cord sectioned dogs given 0.2 mg. of endotoxin become hypotensive as do normal animals. Reference to Figure 1 indicates that even with a dose of endotoxin which consistently produced adrenal medullary stimulation in normal dogs (Figure 2), there was no response in the cord sectioned animals. One cord sectioned animal is not included on the protocol because he

TABLE III

Output per minute of E–NE and 17-OH from right adrenal gland after endotoxin in cord sectioned dogs *

Experiment no.	Substance measured	Dose endotoxin	Control values	Minutes†	Adrenal venous output (μg./min.) 10	20	30	40	50	60	75	90	105	120	150	180	210	240
		mg.																
1B	17-OH	0.01	0.7		2.8		6.8	13.1			14.1		25.0	26.4	22.9	21.8	18.4	21.7
	E–NE		0.02		0.02		0.05	0.03			0.05	‡	0.01	0.04	0.01	0.02	0.02	0.02
2B	17-OH	0.01	0.02			7.3	143	17.7		15.0	14.5		11.2	10.5	14.0	12.1		12.9
	E–NE		0.00			0.01	0.00	0.01		0.02	0.02	‡	0.01	0.02	0.01	0.01	0.01	0.01
3B	17-OH	0.20	3.0			17.8	16.8		11.0	12.6	9.4	18.1			16.2			
	E–NE		0.08			0.12	0.13		0.08	0.08	0.06	0.06			0.03			

* The rectal temperature of all three dogs rose at least 1° C. after endotoxin.
† Time after endotoxin administration.
‡ 0.2 mg. endotoxin I.V.

died following the injection of 0.2 mg. of endotoxin. Two of the other cord sectioned animals became very sick, with vomiting and hypotension following 0.01 mg. of endotoxin and it must be concluded that spinal sectioned animals are more sensitive than normal animals to these effects of endotoxin administration.

DISCUSSION

It is possible, without considering the data presented in this report, to make out quite a convincing case for the participation of epinephrine in the systemic response to bacterial endotoxin. Endotoxin sensitizes blood vessels to the action of epinephrine and norepinephrine (7). Intradermal injection of epinephrine in rabbits either preceded or followed by intravenous endotoxin yields an area of hemorrhagic necrosis where epinephrine was injected (8). Treatment with adrenolytic substances such as dibenamine prevents the vascular response of rabbits to typhoid endotoxin (9). Boquet and Izard conclude that endotoxin leads to the liberation of epinephrine or epinephrine-like substances which themselves lead to peripheral vasoconstriction. Adrenolytic agents should therefore prevent the blood vessel constriction which occurs following endotoxin administration. Feldman and Gellhorn (10) suggest that fever and hyperglycemia following endotoxin administration are due to stimulation of the sympathico-

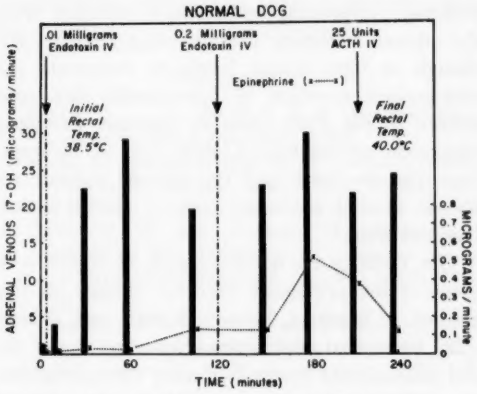

FIG. 1. DOG WITH SPINAL CORD TRANSECTION AT C-7 GIVEN 0.01 AND 0.2 OF ENDOTOXIN INTRAVENOUSLY

There is no epinephrine response in this animal to either the small or large dose of endotoxin, although increased adrenal cortical secretion and fever occur.

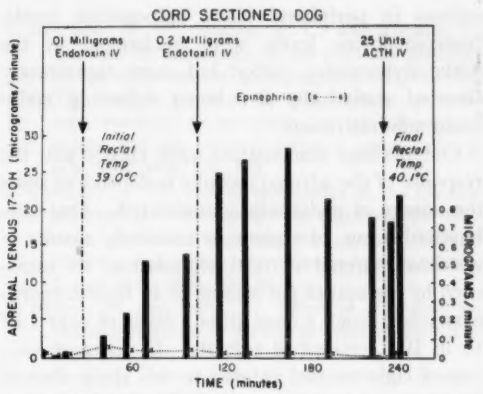

FIG. 2. NORMAL DOG GIVEN 0.01 MG. ENDOTOXIN INTRAVENOUSLY, FOLLOWED BY 0.2 MG. TWO HOURS LATER

Note significant epinephrine response following the larger dose of endotoxin.

adrenal system. In a recent article Thomas, Zweifach and Benacerraf (11) emphasized the possible role of epinephrine in mediating endotoxin effects and they mentioned the further possibility that endotoxin lesions may be due to serotonin.

There are few previous studies related to the problem of adrenal medullary secretion following endotoxin administration. Cannon and Pereira (12) demonstrated that the administration of typhoid bacilli to adrenal-demedullated animals resulted in a different series of events than in animals with normal adrenals. Adrenal-demedullated animals demonstrate a slow secondary rise in temperature in contrast to the biphasic response of the normal animals. Removal of the adrenal medulla rendered the animals more susceptible to the lethal effect of bacterial endotoxin and the above-mentioned authors concluded that "the response of the adrenal medulla during toxic fevers is serviceable to the organism" (12).

Hoshi (13) claimed to have demonstrated increased circulating blood levels of epinephrine with a maximum response two to four hours after the injection of dysenteric toxin. He claims to have observed a depression in blood level of epinephrine following large quantities of the toxin. Blood sugars were measured in Hoshi's experiments and hyperglycemia occurred at the same time as did the increase in blood levels of epinephrine. More recently, Heiffer, Mundy and Grimm (14) have demonstrated that *E. coli* endotoxin given in lethal doses to rabbits resulted in unstatistical ele-

vations in peripheral blood epinephrine levels. Norepinephrine levels were unchanged in the initial hypotensive period but were significantly elevated statistically five hours following endotoxin administration.

Our findings demonstrate quite clearly that the response of the adrenal medulla is dependent upon the dosage of endotoxin administered. One one-hundredth mg. of endotoxin uniformly results in maximal adrenal cortical stimulation, as measured by the output per minute of 17 hydroxycorticosteroid. Such a dose gives a fever of over 1.0° C. in 100 per cent of animals. In contrast, only two of eight normal animals in this study showed an increased secretion of E–NE following a dosage of 0.01 mg. of endotoxin. All animals demonstrated an adrenal medullary response to a dosage of 0.1 mg. or greater. Therefore, although it is possible that at higher dosage levels the endogenous release of epinephrine from the adrenal medulla accounts for some of the systemic results after bacterial endotoxin administration, the occurrence of these effects at lower dosage levels is not dependent upon epinephrine. It is interesting that the response of the adrenal medulla is one of the least sensitive indices of endotoxin activity, in contrast to the extreme sensitivity of the pituitary-adrenal axis.

Results of the experiments in the cord sectioned animals show that the mechanism of endotoxin action on the adrenal medulla at high dosage levels is not a direct one and that it is not a result of hypotension. If endotoxin passed via the blood stream to the adrenal medulla and directly stimulated the release of epinephrine and norepinephrine, spinal cord section should not alter this effect. The fact that no adrenal medullary output increase occurred in response to large doses of bacterial endotoxin in hypotensive cord sectioned animals indicates that nervous pathways are necessary to this response. It does not give evidence as to whether the essential nervous connections are afferent or efferent. It seems most likely, however, that endotoxin acts at some site in the central nervous system above the level of cord transection with stimulation of nerve fibers passing down the cord and out the splanchnic nerves which supply the adrenal medulla. That the adrenal cortical stimulating effect does not depend upon descending nerve pathways is shown by the maximal adrenocortical response to endotoxin administration seen in the cord sectioned animals. The great potency of intrathecally administered endotoxin in producing fever and other systemic responses has been emphasized by Keene (15), Bennett and Cluff (1) and others.

There appear to be several pathways by which stimulation of epinephrine release from the adrenal medulla may occur. von Euler (16) describes experiments in which splanchnic nerve stimulation, intravenously administered nicotine and histamine all result in high levels of peripheral blood epinephrine. Several investigators have described experiments involving central nervous system stimulation and its effect upon epinephrine release from the adrenal medulla. Centers exist in the cerebral cortex (17, 18) and hypothalamus (19) which both increase and decrease adrenal medullary epinephrine secretion and it is reported that ablation of the frontal cortex results in altered adrenal medullary secretion. Our studies indicate that the administration of endotoxin results in adrenal medullary stimulation as a result of a direct action on the central nervous system. Experiments in dogs with "isolated pituitary islands" indicate that the central nervous system center for epinephrine secretion is located in the high medulla or pons (20). The epinephrine center evidently acts on the adrenal medulla through descending nervous pathways in the spinal cord. Suzuki and co-workers (21) have described descending spinal pathways subserving epinephrine secretion from the adrenal following insulin hypoglycemia. Although at high dosage levels of endotoxin the endogenous secretion of epinephrine may contribute to the total systemic response following endotoxin administration, there can be no question that the fever and the adrenal cortical response, at least, are independent of adrenal medullary activation.

Our findings are not completely in accord with some of the previously reported studies on this subject. Chambers, Koenig, Koenig and Windle (22) found that dogs with cord section at C-7 did not demonstrate fevers following the administration of pyromen. Keeton (23), however, found that although animals given typhoid vaccine failed to develop fever two to four days after cord section, if the period after transection were extended to three to seven days, fever did occur. In the

present study it was found that dogs responded to endotoxin with fever as early as one day after cord transection and that this fever producing effect of endotoxin was still present as long as three weeks after cord transection.

SUMMARY

1. Small doses of *E. coli* endotoxin (0.01 mg.) administered intravenously to dogs result in maximal adrenal cortical stimulation and fever, but infrequently lead to increased adrenal medullary secretion.

2. Large doses of *E. coli* endotoxin (0.2 mg.) administered intravenously to dogs result in adrenal medullary stimulation, in addition to the adrenal cortical stimulating and fever promoting activities.

3. Transection of the spinal cord at C-7 abolishes the adrenal medullary response to large doses of bacterial endotoxin, leaving unimpaired the febrile, hypotensive and adrenal cortical responses. It is suggested that adrenal medullary activation following endotoxin is dependent on descending nerve pathways in the spinal cord.

4. Epinephrine release is not necessary for the febrile and adrenocortical stimulating effects of endotoxin.

5. It is concluded that there is a differential response of the adrenal cortex and medulla to endotoxin, with the pituitary adrenal axis more sensitive than the central nervous system center which controls epinephrine release.

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RELATIONSHIP BETWEEN A RANGE OF TISSUE TEMPERATURE AND LOCAL OXYGEN UPTAKE IN THE HUMAN FOREARM. III. CHANGES OBSERVED AFTER ANAEROBIC WORK, IN THE POSTEXERCISE PERIOD*†

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It is generally accepted that a blood flow debt is incurred in active muscles which is repaid in the postexercise period through the production of a hyperemia, the magnitude of the latter being related to the concentration of local metabolites at the end of the period of work (1–3). However, it has been shown by Barcroft, Dornhorst, McClatchey and Tanner (2) that increasing the blood available to the forearm by release of sympathetic vasoconstrictor tone had no effect upon the size or rate of repayment of the blood flow debt. Patterson and Shepherd (3) have reported similar results after the amount of metabolically available blood passing through the tissues was doubled by means of arterial infusion of vasodilator substances. Such evidence has been interpreted as indicating that augmenting the resting circulation alone has no influence on the rate of inactivation or removal of metabolites formed in exercising muscles.

The purpose of the present investigation was to determine whether or not an increase in blood flow and tissue temperature produced by local application of heat had any effect upon the magnitude of the oxygen debt incurred in the forearm during a short period of anaerobic work. This approach was undertaken because of the data previously obtained indicating that the resting oxygen uptake (4) and the oxygen debt resulting from five minutes of arterial occlusion (5) were increased by raising tissue temperatures and decreased by lowering them. Anaerobic exercise was used in order to ascertain accurately the entire oxygen debt through a study of the excess oxygen repayment in the postexercise period.

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METHOD

Fourteen experiments were performed on 14 healthy subjects between the ages of 21 and 34 years. The duration of the period of observation averaged six hours, during which time food intake was limited to one or two small chocolate bars.

Utilizing the same technique and environmental conditions described in a previous paper (4), the following measurements were made: blood flow, oxygen content of venous blood and muscle,[1] subcutaneous tissue and skin temperatures, all obtained from the upper two-thirds of the forearm; blood pressure and pulse rate readings, using the opposite limb; and determinations of rectal temperature. In all instances blood samples were collected from a vein in the antecubital fossa which had no obvious superficial tributaries and which appeared to drain deep tissues primarily. The polyethylene catheter was passed far enough into the vessel, in the direction of the hand, so that its tip lay in the mass of tissue enclosed by the plethysmograph.

Plan of experiment. After the segment of forearm was placed in the plethysmograph and the openings made watertight (6), the machine was filled with water at either 45° or 28 to 30° C. (bath temperature). Then the subject was instructed to compress a blood pressure bulb 60 times in 30 seconds, to raise the pressure in a five gallon bottle to a level of 50 to 80 mm. Hg, the height reached depending, in part, upon his state of physical fitness. During this time blood was prevented from entering the forearm through the application of an arterial occlusion pressure to the arm. No records were made in the postexercise period, since the purpose of the initial trial was to acquaint the subject with the type of exercise used in the study.

After a period of more than 90 minutes of exposure

[1] The muscle thermocouple was inserted perpendicularly to the skin into the brachioradialis muscle mass for an average distance of 4.1 cm. (range 2.1 to 5.3 cm.).

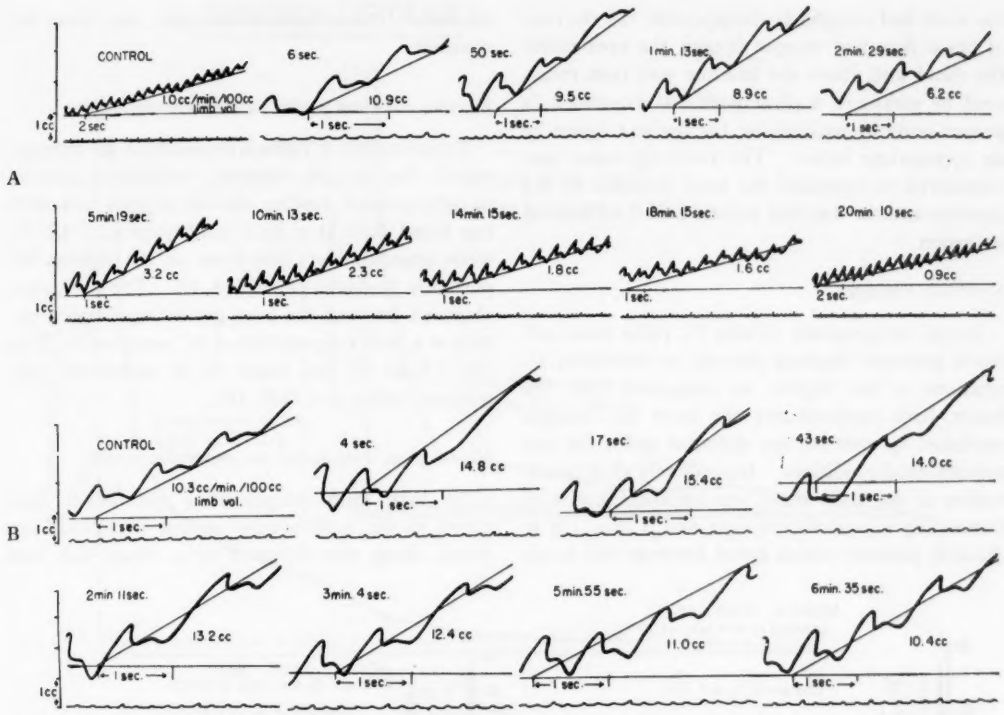

FIG. 1. FOREARM BLOOD FLOW CURVES OBTAINED ON SUBJECT J. F. BEFORE AND AFTER ONE-HALF MINUTE OF ANAEROBIC EXERCISE

A = control and postexercise readings collected at a bath temperature of 30° C. B = control and postexercise readings collected at a bath temperature of 45° C. The calculated blood flow in ml. per minute per 100 ml. limb volume and the time in the postexercise period are indicated for each determination. Calibration scale, in cc. divisions, is to the left of the curve. An arterial occlusion pressure was maintained at the wrist during blood flow recordings.

of the forearm to the maintained water temperature, control readings for blood flow, venous oxygen content and tissue temperatures were collected. Following this another one-half minute period of anaerobic exercise was performed, during which time the stopcock attached to the catheter in the vein remained open to permit drainage of blood out of the vessel. Immediately after the termination of the exercise and the release of the arterial occlusion pressure on the arm, blood flow readings (Figure 1), venous blood samples and temperature recordings were obtained at rapid intervals, generally three or four in the first minute of the postexercise period, two in the second and five spaced over the next 10 to 20 minutes. In every instance care was taken not to draw a blood sample at the same time as blood flow was being recorded (5).

After the various readings had returned to the preexercise baseline, the bath temperature was changed to either the higher or the lower one, depending upon which had been used initially [2] and the procedure already described was repeated. The level to which the pressure was raised in the five gallon bottle by the exercise was kept the same as in the previous trials.

[2] The order in which the experiments were performed in no way affected the results.

RESULTS

From the venous oxygen determinations and the derived figures for oxygen content of arterial blood, oxygen arteriovenous difference was obtained for the resting and for the postexercise periods. On the basis of these figures and the readings of blood flow, oxygen uptake was then calculated, using the Fick principle (7).

From the data, graphs were constructed for postexercise blood flow, oxygen arteriovenous difference and oxygen uptake (Figures 2 and 3). In each instance the values were plotted against time in minutes, with the baseline being derived from three to five pre-exercise control readings and from a similar number obtained after the effect of

the work had completely disappeared. In the case of blood flow and oxygen uptake, the area under the curve and above the baseline was then measured by means of a planimeter and expressed in proper units by multiplying the derived figure by an appropriate factor. The resulting value was considered to represent the total response to the exercise and the one-half minute period of arterial occlusion.

Systemic changes

Rectal temperatures (Table I), pulse rates and blood pressure readings showed no consistent alterations at the higher, as compared with the lower, bath temperature; nor were the changes produced by exercise any different under the two experimental conditions. Immediately after termination of the work there was an average rise of 9 mm. Hg in systolic pressure and 3 mm. Hg in diastolic pressure which lasted for from two to six minutes. The alterations in pulse rate were inconstant.

Results obtained under resting conditions

Examination of Table I reveals that the average values for oxygen capacity, calculated arterial oxygen content, resting venous oxygen and resting blood flow at a bath temperature of 45° C. were approximately the same as the figures reported in previous papers (4, 5). The difference observed between the average resting oxygen uptake at a bath temperature of 45° and that at 28 to 30° (Table I) was found to be statistically significant, with a $p < 0.01$ (8).

Blood flow responses to anaerobic work

At both bath temperatures the blood flow curve in the postexercise period manifested an initial sharp rise followed by a sharp fall, and

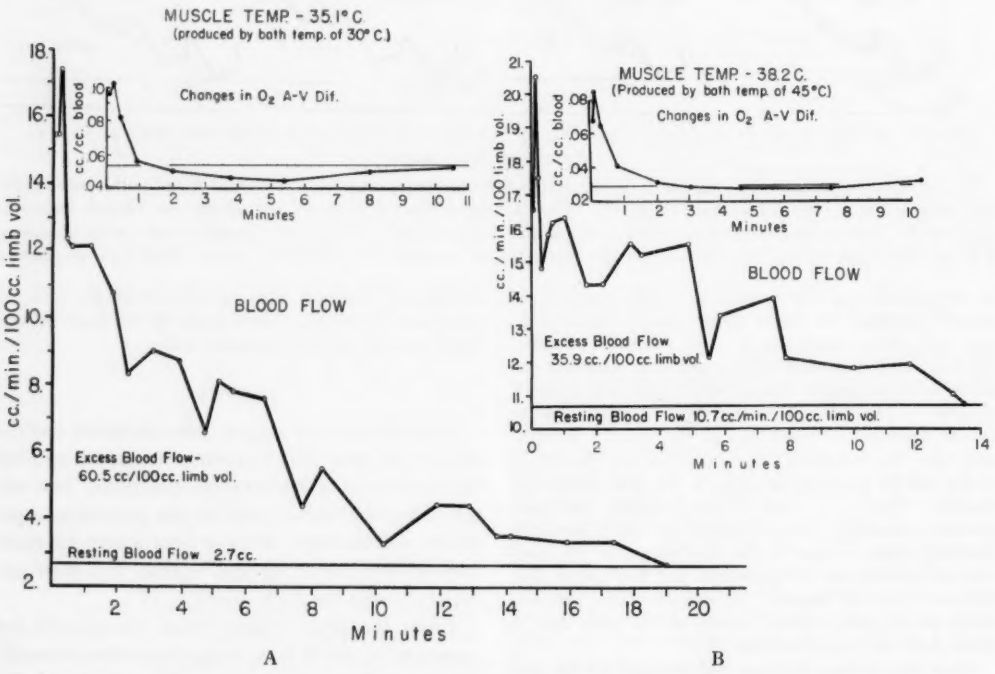

FIG. 2. GRAPHS OF BLOOD FLOW AND OF OXYGEN ARTERIOVENOUS DIFFERENCES OBTAINED IN THE POSTEXERCISE PERIOD ON SUBJECT E. P.

A = bath temperature of 30° C. B = bath temperature of 45° C. A negative deflection follows the positive one in the graph for oxygen arteriovenous difference obtained at a bath temperature of 30° C. This is not observed at a bath temperature of 45° C.

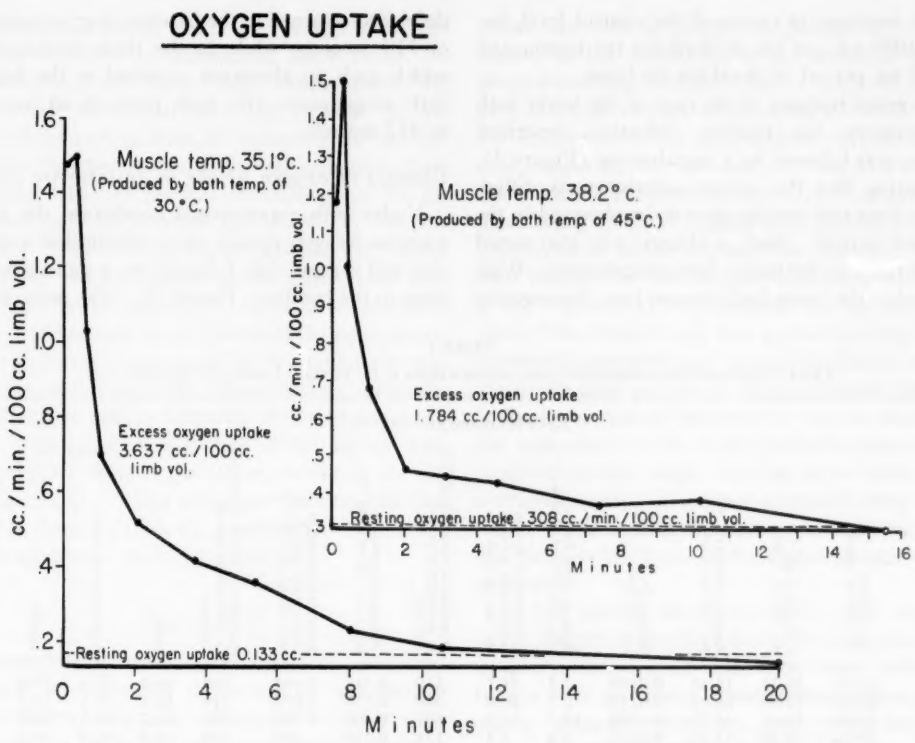
OXYGEN UPTAKE
Muscle temp. 35.1°c.
(Produced by bath temp. of 30.°C.)
Excess oxygen uptake
3.637 cc./100 cc. limb vol.
Resting oxygen uptake 0.133 cc.
cc./min./100 cc. limb vol.
Minutes
Muscle temp. 38.2° c.
(Produced by bath temp. of 45°C.)
Excess oxygen uptake
1.784 cc./100 cc. limb vol.
Resting oxygen uptake .308 cc./min./100 cc. limb vol.

FIG. 3. GRAPH OF OXYGEN UPTAKE OBTAINED IN THE POSTEXERCISE PERIOD ON SUBJECT E. P.
Lower curve = bath temperature of 30° C. Upper curve = bath temperature of 45° C. Graphs are based on blood flow and oxygen arteriovenous differences as depicted in Figure 2.

then a more gradual drop to the baseline (Figure 2). The peak of the response occurred early in the postexercise period (average of 15 seconds at the higher bath temperature and 22 seconds at the lower). The actual maximal increase, in excess of the resting level, was generally larger at the lower bath temperature (average of 9.1 ml. per minute per 100 ml. limb volume, as compared with 6.9 ml. for the higher bath temperature). Furthermore, the average duration of the postexercise excess blood flow response was longer, 902 seconds as compared with 538 seconds for the higher bath temperature, a difference which was found to be statistically significant ($p < 0.01$). Finally, the total augmentation in blood flow, in excess of the resting level, in the postexercise period was also greater at the lower bath temperature except in four cases. Of the latter, in two the response was approximately the same, while in the remaining two it was greater at the higher bath temperature. The difference between the two sets of readings (Table I) was found to be statistically significant, with a $p < 0.01$.

Changes in oxygen arteriovenous difference in postexercise period

Consistently at both bath temperatures definite increases in oxygen arteriovenous difference were noted in the initial portion of the postexercise period. The resulting curve (Figure 2) consisted of a sharply rising slope, followed by a sharply falling one, with the baseline being reached at an average of 124 and 175 seconds after termination of exercise for the lower and higher bath temperatures, respectively. The greatest increase in oxygen arteriovenous difference occurred early (average of 19 seconds for the lower and 16 seconds for the higher bath temperature) the actual av-

erage increase, in excess of the control level, being 0.058 ml. per ml. of blood for the former and 0.082 ml. per ml. of blood for the latter.

In every instance in the case of the lower bath temperature, the positive deflection, described above, was followed by a negative one (Figure 2), indicating that the oxygen arteriovenous differences were now smaller than those observed in the control period. Such a change was also noted three times at the higher bath temperature. With regard to the lower bath temperature, the negative deflection returned to the baseline after an average of 710 seconds, while in the three instances in which such an alteration occurred at the higher bath temperature, this took place at an average of 432 seconds.

Changes in oxygen uptake in postexercise period

Under both experimental conditions, the postexercise oxygen uptake curve manifested a rapid rise and a rapid fall, followed by a more gradual drop to the baseline (Figure 3). The peak of the

TABLE I

Effect of different bath temperatures on oxygen uptake of the forearm following anaerobic work

Subject	O_2 capacity *ml./100 ml. blood*	Calculated art. O_2 *ml./100 ml. blood*	Resting venous O_2 *ml./100 ml. blood*	Resting O_2 A.V. diff. *ml./ml. blood*	Resting blood flow *ml./min./100 ml. limb vol.*	Excess blood flow in postexercise period *ml./100 ml. limb vol.*	Duration of excess blood flow in postexercise period *(seconds)*	Resting O_2 uptake *ml./min./100 ml. limb vol.*	Excess O_2 uptake in postexercise period *ml./100 ml. limb vol.*	Duration of excess O_2 uptake in postexercise period *(seconds)*	Temp. ° C. Skin	Subcutaneous tissue	Muscle	Rectal
Bath temperature—45° C.														
P. S.	21.12	20.34	18.44	0.0190	7.5	31.7	510	0.142	1.468	565	40.9	39.4	39.0	
G. W.	19.84	19.09	16.92	0.0217	6.2	20.0	360	0.134	2.078	340	40.6	39.6	38.2	
E. P.	19.39	18.65	15.75	0.0289	10.7	35.9	807	0.309	1.784	929	41.5	40.1	38.2	
G. P.	19.24	18.50	13.23	0.0527	3.4	2.3	175	0.179	.453	170	42.5	42.5	39.3	36.6
S. M.	19.13	18.39	12.74	0.0564	6.2	10.3	327	0.350	.479	146	40.2	40.2	38.1	37.4
N. D.	23.82	22.99	20.12	0.0286	10.5	3.5	186	0.300	1.475	302	40.5	39.7	38.6	36.9
M. I.	16.60	15.91	12.83	0.0308	8.8	30.6	615	0.271	1.493	357	42.3	40.1	37.7	37.5
N. S.	22.22	21.42	18.95	0.0247	9.9	34.7	813	0.244	3.617	794	40.8	39.5	37.8	37.4
G. L.	22.50	21.69	14.93	0.0675	8.9	15.4	552	0.601	1.765	615	42.0	39.4	37.2	37.1
S. S.	21.25	20.48	15.49	0.0498	6.9	3.6	272	0.344	.910	208	40.1	41.1	37.8	37.6
J. T.	20.43	19.66	18.09	0.0156	6.2	25.0	1,020	0.097	.545	272	42.0	40.1	38.3	37.6
J. F.	22.37	21.56	18.01	0.0354	10.0	20.3	530	0.354	.645	380	41.4	40.1	38.6	37.8
A. F.	20.34	19.57	17.95	0.0162	8.0	20.8	330	0.130	2.642	567		40.7	38.2	37.7
C. P.	18.15	17.43	16.15	0.0127	8.3	37.8	1,030	0.105	1.840	701	41.1	40.6	38.3	37.6
Mean	20.46	19.69	16.40	0.0329	8.0	20.8	538	0.254	1.514	453	41.2	40.2	38.2	37.4
Standard error					±0.55	±3.33		±0.037	±0.240					
Bath temperature—28–30° C.														
P. S.	21.12	20.34	13.63	0.0671	1.0	55.1	1,165	0.067	3.792	1,226	30.2	31.2	33.6	
G. W.	19.84	19.09	12.48	0.0660	1.1	21.1	367	0.073	2.141	400	28.9	31.7	36.3	
E. P.	19.39	18.65	13.25	0.0539	2.5	60.5	1,164	0.135	3.637	1,205	34.9	33.3	35.1	
G. P.	19.24	18.50	10.11	0.0838	1.5	17.8	549	0.126	1.921	1,045	30.3	31.6	34.3	36.7
S. M.	19.13	18.39	11.98	0.0641	1.8	35.2	860	0.115	.751	510	33.4	31.0	36.0	37.1
N. D.	23.82	22.99	15.81	0.0717	2.2	14.9	462	0.157	1.324	785	31.4	32.6	36.4	37.0
M. I.	16.60	15.91	9.44	0.0646	1.1	30.4	640	0.071	2.454	375	30.3	31.5	35.7	37.3
N. S.	22.22	21.42	12.08	0.0933	1.2	34.9	1,105	0.112	3.637	1,214	29.4	29.9	33.5	37.4
G. L.	22.50	21.69	11.21	0.1048	1.6	29.2	1,068	0.168	2.296	725	29.9	31.1	34.1	36.9
S. S.	21.25	20.48	11.73	0.0875	1.9	56.8	1,257	0.166	2.308	420	31.2	31.5	35.2	37.3
J. T.	20.43	19.66	13.76	0.0590	2.0	18.1	987	0.118	1.154	370	30.0	31.4	34.9	37.5
J. F.	22.37	21.56	12.18	0.0937	1.1	40.9	1,195	0.103	2.328	1,149	29.0	29.1	34.8	37.7
A. F.	20.34	19.57	12.68	0.0689	2.4	75.8	1,087	0.165	6.132	1,229	30.7	31.4	35.4	37.6
C. P.	18.15	17.43	10.01	0.0742	1.3	32.4	727	0.096	2.404	1,255	29.9	30.0	33.9	37.4
Mean	20.46	19.69	12.17	0.0752	1.6	37.4	902	0.119	2.591	851	30.7	31.2	34.9	37.3
Standard error					±0.14	±4.9		±0.010	±0.338					

response was noted within an average of 13 and 15 seconds after termination of the exercise, for the higher and lower bath temperatures, respectively, the actual maximal increase, in excess of the resting level, also showing no real difference for the two experimental conditions (average of 1.162 ml. per 100 ml. limb volume for the higher bath temperature and 1.147 ml. for the lower). However, in every instance the control level was reached sooner at the higher bath temperature, an average of 453 seconds as compared with 851 seconds at the lower bath temperature. This difference was found to be statistically significant, with $p < 0.001$. Furthermore, when the total oxygen uptake, in excess of the resting level, was determined, it was less at the higher bath temperature than at the lower, except in one instance (N.D.). The difference between the two sets of figures (Table I) was found to be statistically significant, with a $p < 0.01$.

DISCUSSION

Examination of the data on blood flow and oxygen arteriovenous difference reveals that in all instances at the two bath temperatures the oxygen debt in the initial portion of the postexercise period was repaid both by a marked increase in blood flow and by a greater than normal extraction of oxygen from each ml. of blood (Figure 2). This combined response, which lasted for only two to three minutes, was then followed by a much longer period of augmentation of local circulation, associated with either a normal or an elevated oxygen content of venous blood. Such observations are not in accord with the findings of Quensel and Kramer (9) and of Love (10), who noted a substantial increase in oxygen content of the effluent venous blood immediately after termination of a period of tetanic or rhythmic contractions. It is possible that such a difference in results can be attributed to the use of aerobic exercise by these authors and anaerobic exercise in the present study.

In this investigation, the consistent finding at the lower muscle temperature and the occasional one at the higher, of smaller than control oxygen arteriovenous differences in the later portion of the postexercise period can be interpreted as indicating that during this interval the increase in blood flow was of greater magnitude than warranted for the satisfaction of the metabolic needs of the exercising muscles. Such a response could, in a sense, be considered an inefficient means for the repayment of the oxygen debt. At the same time, however, it is necessary to note that at the lower muscle temperature, the oxygen uptake figures, based on these smaller than control oxygen arteriovenous differences and the increased blood flow values, were still greater than those obtained at the higher muscle temperature in the same period. This would imply that part of the large and prolonged augmentation in local circulation existing in the later portion of the postexercise period at the lower muscle temperature was required for the repayment of the more marked oxygen debt incurred by the work. On the other hand, the average quantity of oxygen extracted from each ml. of blood during the postexercise period was less than in the case of the higher muscle temperature.

As has already been mentioned, in all experiments the blood samples used in the calculation of oxygen arteriovenous difference were collected from a vein apparently draining deep tissues, primarily, muscle. However, since there was no accurate means of ascertaining whether or not such a vessel received tributaries from superficial veins distal to its subcutaneous location, it is necessary to consider the question of contamination of the deep venous blood sample and the possible effects this might have had on the results.

As pointed out in a previous paper (4), resting oxygen uptake figures at a bath temperature of 45° C., based on oxygen determinations of venous blood obtained from superficial tissues, were smaller than those derived from samples collected from deep veins. It would be expected, therefore, that in this study, at the higher bath temperature the presence of superficial venous blood in the sample would have lowered the level of the theoretical resting oxygen uptake during the postexercise period, thus increasing the magnitude of the excess oxygen uptake elicited by the exercise. Since such a situation did not exist at the lower bath temperature (4), any error resulting from contamination of the blood sample would have tended to make the calculated difference between the postexercise oxygen uptakes for the two experimental conditions less than the true value.

It is also of interest to point out that at the two bath temperatures, contamination of the venous sample might not have had the same effect on the contribution resulting from the one-half minute of arterial occlusion. In this respect it should be mentioned that at a bath temperature of 45° C., with the cutaneous and subcutaneous vessels already almost fully dilated by the heat, only minimal vascular changes would be occurring in the skin and subcutaneous tissue during reactive hyperemia. As a result, the increased needs of these structures, produced by the period of anoxia, would have to be satisfied almost solely through the mechanism of extraction of oxygen from each ml. of blood. Consequently, it would be expected that during reactive hyperemia, oxygen arteriovenous difference, and hence, oxygen uptake, based on blood from superficial tissues alone, would be greater than at the lower bath temperature, under which circumstances the cutaneous and subcutaneous vessels were still capable of dilating in this period. Therefore, any contamination of the deep blood sample would again tend to make the difference in calculated postexercise excess oxygen uptake at the two bath temperatures less than the true value.

Another factor which must be evaluated is the effect of the one-half minute period of arterial occlusion utilized during the work. In this regard it should be mentioned that at both bath temperatures, the changes in the various readings following termination of the physical effort and removal of the arterial occlusion pressure were a composite of the alterations resulting from the state of anoxia and the exercise, with the contribution of the former being relatively negligible because of the short period of arterial occlusion. Nevertheless, it is necessary to know whether or not the change due to anoxia alone was of equal magnitude at the two bath temperatures. Of interest in this respect is the observation in a previous study (5) that the oxygen repayment following a five minute period of arterial occlusion, in excess of the baseline, was significantly greater at a higher than at a lower tissue temperature. On the basis of such findings, it would be safe to assume that if there had been a difference in the response to the one-half minute of arterial occlusion at the two bath temperatures, this would have manifested itself in the form of an increase in the excess oxygen uptake at the higher level, thus making the calculated difference between this figure and the one obtained at the lower bath temperature less than the true value.

One must also consider the question of the blood trapped in the forearm with the sudden application of the arterial occlusion pressure, since as a result, a source of tissue oxygen was conceivably available during the period of anaerobic work. Although this possibility existed for both bath temperatures, one would expect a larger amount of blood to be found in the vascular tree at the higher level because of the marked vasodilatation and augmentation of blood flow produced by the heat. This could have been reflected in the repayment of a smaller oxygen debt in the postexercise period, thus tending to make the calculated difference between the postexercise excess oxygen uptake at the two bath temperatures greater.

That the above objection did not particularly apply to the present study is supported by the following points: First, the vasodilatation at the high bath temperature occurred primarily in the cutaneous and subcutaneous tissues, so that most of the increase in the volume of trapped blood was not available to the active muscles. Furthermore, it can be assumed that under both experimental conditions, as a result of the pumping action of the exercising muscles, there was a rapid movement of blood from the arterial tree into the venous system, where it was no longer utilizable as a source of tissue oxygen. Finally, by maintaining the stopcock to the catheter in the deep vein open during the entire period of work, drainage of blood out of the muscles was facilitated, thus reducing further the possibility of aerobic metabolism during the exercise. It is realized, of course, that this means was not available for all portions of the forearm, except insofar as blood from them was shunted into the cannulated vein, since this vessel, through its opening to the environment, now represented a focus of reduced peripheral resistance. Another supporting point for the view that the trapped blood was not an important source of oxygen is the finding in a previous study (5) that the oxygen debt incurred during the five minute period of arterial occlusion and repaid subsequently was significantly greater at the higher tissue temperature than at the lower.

On the basis of the data obtained in the pres-

ent study, it can therefore be stated that artificially raising the temperature of the muscles of the forearm caused a reduction in the magnitude of the oxygen debt incurred during anaerobic work. Such a finding tends to support the belief that there are some beneficial effects from the "warming-up" exercises carried out by athletes preliminary to the actual work performance (11–13), a view which has recently been questioned (14).

SUMMARY AND CONCLUSIONS

Using venous occlusion plethysmography and studies of local oxygen arteriovenous difference, the effect of altering external temperature on oxygen uptake of the forearm musculature following anaerobic work was investigated in a series of 14 normal subjects.

1. It was found that at both the higher and lower muscle temperatures the oxygen debt incurred during anaerobic work was repaid in the early postexercise period by an increase in blood flow and by a greater than normal extraction of oxygen, and in the later and more protracted period, by an augmentation in circulation alone.

2. At the higher muscle temperature the blood flow response to the exercise was less marked than at the lower, but oxygen extraction per ml. of blood was greater.

3. At the higher muscle temperature, the postexercise oxygen uptake was reduced, despite an increase in resting oxygen uptake.

These results tend to support the belief that there is some basis for the use of local heat preceding the exercise of limbs in physical medicine and for the "warming-up" procedure customarily practiced by athletes.

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EXPERIMENTAL POTASSIUM DEPLETION IN NORMAL HUMAN SUBJECTS. I. RELATION OF IONIC INTAKES TO THE RENAL CONSERVATION OF POTASSIUM*

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When healthy human subjects ingest a diet deficient in potassium but not in calories and protein, the daily loss of potassium in the urine progressively decreases (1–7). Since the daily urinary loss often continues to exceed the daily intake (2, 3, 6), prompt and effective renal conservation of potassium has been questioned. Furthermore, the fecal excretion of the ion has been found to exceed the intake when the latter is low (2). But these studies do not indicate to what level the intake of potassium must fall before the renal excretion and the fecal excretion consistently exceed the intake and lead to a progressive deficit of the ion. Nor do these studies completely define the conditioning effects of intakes of other major ions on the excretion of potassium during varying degrees of potassium deprivation.

The aim of this paper is to report quantitative data describing the loss of potassium in the urine and feces of human subjects while ingesting low potassium diets and to discuss certain metabolic corollaries of the loss of potassium in urine and feces relevant to the physiological regulation of potassium excretion.

EXPERIMENTAL PROCEDURE AND METHODS

Human subjects. Fourteen balance studies were done in 11 volunteer male subjects (nine medical students and the authors). By the criteria of the medical history and physical examination every subject appeared to be in good health. However, Subject T. C. was subsequently shown by roentgen examination to have an asymptomatic duodenal ulcer.

The study design. The basic study plan consisted of a control period lasting three to eight days, and a potassium depletion period lasting six to 21 days. The duration of each study largely depended upon how long the subject could remain on the Metabolic Unit or tolerate the dietary restriction. During the control period a measured "normal diet" was ingested. Potassium depletion was studied at three levels of potassium intake: 25 to 27 mEq. per day, 14 to 16 mEq. per day, and less than 1 mEq. per day.

Experiments were designed to study, in the depletion period, the effect on urinary and fecal potassium loss of: *1*) a normal or high sodium intake throughout the entire study; *2*) ingestion of sodium and potassium with chloride or with chloride and bicarbonate in a ratio of 3:1; *3*) a low intake of 14 mEq. of sodium per day during the depletion period; and *4*) progressively increasing oral doses of KCl to more than 300 mEq. per day in the control period.

The renal conservation of potassium and sodium was compared in separate studies in which the intake of either ion averaged 15 mEq. per day.

Potassium skin loss was not measured in our subjects; such loss of potassium during normal potassium intakes amounts to about 4 mEq. per day (8, 9). No correction for skin loss was made.

Subjects were allowed normal activity in the hospital and medical school during the cool fall and winter months. Studies conducted during the warm spring and summer months took place in the air-conditioned Metabolic Unit or air-conditioned portions of the hospital. Balance periods lasted 24 hours, starting at 7 a.m. The subject was awakened at that time and voided urine immediately. He was then weighed, and blood was drawn. He was then given breakfast.

Urine and stool collection. Urine was voided into a screw-capped, wide-mouthed jar large enough to hold the 24 hour specimen, as well as a measured constant amount of mineral oil and chloroform or toluol. Urine was refrigerated between voidings. Feces were collected for periods from two to eight days according to the study plan. They were passed directly into tared, wax-impregnated cardboard containers which were kept covered and refrigerated when not in use.

The stools in the depletion periods of Subjects L. P., H. F., E. H.$_1$, E. H.$_2$, R. S.$_1$ and R. S.$_2$ were demarcated with either barium sulfate or charcoal.

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A stool-marking agent, charcoal or carmine, was given to Subjects R. A., J. B., J. D., C. P. and J. M. on their arrival on the Metabolic Unit; stool preceding this marker was discarded. The ends of the control period and the depletion period were likewise marked. Two to six day intervals were also marked during the control and depletion periods in some studies. Stool data from only these subjects were used in estimating the daily decline of the measured constituents.

Blood was drawn with minimal stasis and clotting took place under oil at room temperature. Serum was separated anaerobically.

Dietary procedure. Water was allowed *ad libitum* and the amount drunk each day was recorded. Subjects ingested all the food given; when possible, food was eaten from the container in which it was prepared so that the juices and small fragments of food could be completely ingested. Metabolic diets were made up from the following food sources: *1*) weighed portions of previously analyzed foodstuff; *2*) commercial dialyzed milk preparations [Theralac®, Lonalac® (Mead Johnson formula L.P. 412)],[1] and fresh whole homogenized milk passed through a monobed ion exchange resin column (Amberlite MB-3)[2]; *3*) lactose; *4*) the addition of measured quantities of $NaHCO_3$, NaCl, KCl, Ca lactate and NH_4Cl when needed as supplements; and *5*) multivitamin supplements.

Subjects on diets containing about 25 mEq. potassium per day were given diets containing solid food. Those on lower potassium intakes consumed whole milk, lactose and vitamin supplemented diets. The total calories ingested were kept constant throughout each study. In a few cases nitrogen intake was reduced during the depletion period.

Specific methods, chemical determinations and calculations

Diet. Samples of homogenized, whole 24 hour diets were analyzed as well as individual samples of the foodstuff comprising this diet. Whole diet analysis was carried out periodically as a check on the summing of previous food analytical values. Agreement was satisfactory for Na, K and N; Cl was somewhat variable. Diets consisting only of treated whole milk were analyzed daily.

Foods were prepared for analysis by nitric acid digestion (10), except for samples analyzed for total nitrogen by the macro-Kjeldahl method.

Chemical determinations. Sodium and potassium were determined by means of a Barclay internal standard flame photometer by the method of Wallace, Holliday, Cushman and Elkinton (10), serum chloride by the method of Franco and Klein (11) and urine chloride by the method of Harvey as described by Peters and Van Slyke (12). Urinary ammonia was measured by the method of Folin and Bell (13). Urinary titratable acidity was measured by titrating an aliquot to pH 7.4 with 0.01 N sodium hydroxide using a Beckman pH meter to determine the endpoint, venous serum CO_2 by the method of Stadie and Van Slyke (14), urine CO_2 by the method of Peters and Van Slyke (12) and serum and urine creatinine by the method of Bonsnes and Taussky (15). Nitrogen was determined by the macro-Kjeldahl method (12). Finger blood pH and carbon dioxide content were determined by the method of Singer and Hastings (16).

Metabolic calculations, with the few exceptions noted in the Results, were performed as previously described (17).

[1] Mead Johnson formula L.P. 412 was kindly supplied by Dr. Warren Cox, Evansville, Ind.

[2] Amberlite MB-3 was obtained from Fisher Scientific Company, 633 Greenwich Street, New York 14, N. Y.

RESULTS

The observed data are recorded in Tables I to VIII.

Renal potassium excretion during the potassium depletion period

The daily rate of renal potassium excretion on a given day varied with the level of oral sodium intake in the depletion period and with the difference between the oral potassium intake in the control and depletion periods. Higher than normal levels of oral sodium intake tended to increase the rate of potassium excretion and lower than normal oral sodium intake may have reduced the rate of renal potassium excretion during the potassium depletion period. When the oral potassium intake was raised to three or four times the normal level during the control period the rate of renal potassium excretion was initially increased in the depletion period.

The lower the oral potassium intake during depletion the lower the daily urinary potassium content. However, the urinary potassium loss continued to be greater than the oral potassium intake when the intake fell below 14 mEq. per day for up to 21 days in one study.

1. Subjects on normal intakes of sodium (130 mEq. per day). In the two subjects *ingesting 25 to 30 mEq. of potassium per day* (Subjects T. C. and C. $P._1$), the daily rate of urinary excretion of this ion, UV_K, fell to a level equivalent to the intake in four to seven days. In the two different experiments on the same subject (E. $H._1$ and E. $H._2$) *ingesting 14 mEq. of potassium per day*, UV_K fell to 25 and 35 mEq. per day, respectively, on the fourth day and further to 19 and 27 mEq. per day, respectively, on the eighth day; in the experiment of E. $H._2$ the nitrogen intake was lower. In two subjects, J. D. and C. $P._2$ (Figure 1), *ingesting less than 1 mEq. K per day*, the UV_K fell to around 13 to 15 mEq. on the fourth day and 9 mEq. on the eighth day. One of these, C. $P._2$, was maintained for a third week on

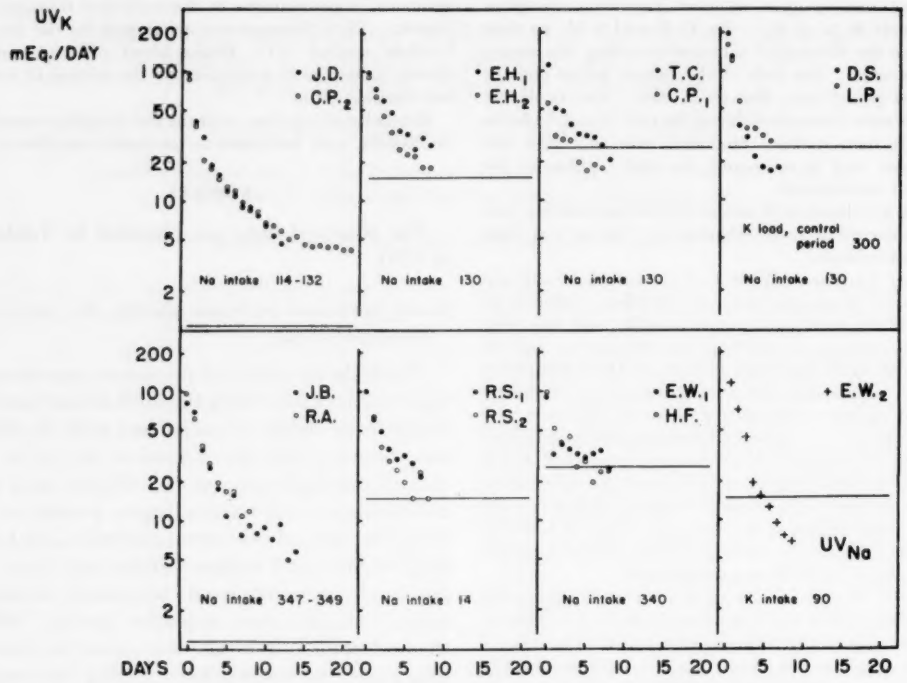

FIG. 1. THE DAILY URINARY EXCRETION OF POTASSIUM OR SODIUM DURING DEPLETION

The values plotted represent the daily urinary excretion of potassium in mEq., except for those of E. W.$_2$ which are for sodium. The first value of each curve represents the subject's average daily excretion during the control period. Depletion intake values are shown by the solid horizontal lines drawn across each graph (Na for E. W.$_2$, K for the rest). Intakes for J. D., C. P.$_2$, J. B. and R. A. were less than 1 mEq. per day. For additional detail on the diets and for discussion, see Table I and text.

potassium deprivation with further diminution of his UV_K to 4.2 mEq. per day. One of these two subjects, J. D., received bicarbonate and chloride in a ratio of 1:3 as the supplemental anions; no difference in the excretory pattern of potassium was observed. The total cumulative amount of potassium lost in the urine showed no relation to the level of potassium intake when compared on the seventh day of depletion. At the 25 to 30 mEq. per day level of intake of potassium Subject C. P.$_1$ lost 138 and Subject T. C. lost 263 mEq. of potassium. Ingesting approximately 14 mEq. of potassium per day, Subject E. H. lost 305 and 232 mEq. potassium during two separate studies. Subjects C. P.$_2$ and J. D. lost 128 and 143 mEq. potassium, respectively, while ingesting less than 1 mEq. potassium per day (Tables I and II).

2. High sodium intake (340 mEq. per day). In two subjects, J. B. and R. A., ingesting less than 1 mEq. K per day, the UV_K fell more slowly, reaching 20 mEq. per day on the fourth day and 10 mEq. on the eighth day (Figure 1). Although J. B. ingested bicarbonate and chloride in a ratio of 1:3 and R. A. ingested only chloride as the supplemental anion, the curve of UV_K was essentially the same in these two subjects.

3. Low sodium diet (14 mEq. per day). The ingestion of a low sodium intake (14 mEq. per day) (R. S.$_1$ and R. S.$_2$) with a potassium intake of 14 mEq. per day made no significant reduction in total urinary loss of potassium when compared to a subject in a normal sodium intake, E. H.$_1$ and E. H.$_2$ (Figure 1).

4. High potassium diet during the control period. In two subjects, D. S. and L. P. (Figure 1), ingesting 300 mEq. potassium per day during the control period and 25 mEq. per day during the

depletion period, the UV_K declined very rapidly from an average control level of approximately 280 mEq. per day to a level of 28 mEq. by the fourth day of depletion. The high initial takeoff of the UV_K curve resulted in a substantially higher urinary potassium loss during the first three days than for the subjects starting from normal levels.

5. Low sodium plus normal potassium intake. One subject, E. W.$_2$, was placed on a diet of 14 mEq. per day of sodium and 90 mEq. per day of potassium, following a control period, for comparison of rate of sodium conservation with a subject, R. S.$_2$, receiving only 14 mEq. per day of each ion. Decrease in excretion rate of sodium was more rapid than that of potassium for comparable levels of intake of the respective ions; the rate of sodium conservation was enhanced by concomitant deprivation of potassium (Figure 2).

Excretion of sodium and other constituents in the urine

Urinary *sodium* excretion tended to decrease during the first three to four days of the period of potassium depletion and then returned to approximately the intake level of sodium. Urinary *chloride* excretion fell abruptly in the same period of K depletion, reflecting the decrease in ingested supplements of potassium chloride. *Phosphate* exhibited no significant variation in excretion. Urinary *nitrogen* excretion for the most part reflected the nitrogen intake. In two of the subjects on less than 1 mEq. potassium

TABLE I

Metabolic data on Subject C. P.$_2$ (normal Na^+ intake)—Intake and urinary output of water, electrolytes, creatinine and nitrogen*

Day	Urine Vol.	pH	S.G.	Na	K	NH_4^+	T.A.	Cl	PO_4	CO_2	N	Cr
	ml.			*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mMoles*	*Gm.*	*mg.*
Control period												
1 C	2,645	5.38	1.010	143	88	66	41	206	37	3.7	16.7	1,706
2 C	2,985	5.00	1.009	161	100	81	50	261	40	2.9	18.4	1,716
3 C	2,540	5.00	1.011	132	100	85	57	225	47	2.8	18.8	1,753
4 C	2,240	4.90	1.011	118	92	85	55	207	44	2.3	19.2	1,769
5 C	2,750	4.88	1.009	122	95	92	53	229	40	2.8	19.1	1,760
Depletion period												
1 D	2,710	5.07	1.008	81	39	90	54	115	47	2.8	18.6	1,748
2 D	2,240	5.30	1.008	53	21	96	49	78	45	2.3	18.0	1,741
3 D	2,680	5.70	1.007	72	18	94	38	90	44	3.1	17.7	1,762
4 D	2,625	5.85	1.006	70	15	90	33	89	38	3.3	16.5	1,752
5 D	2,420	5.80	1.007	78	13	92	35	91	39	3.4	16.5	1,724
6 D	2,560	5.98	1.007	84	12	96	37	112	46	4.5	17.5	1,713
7 D	2,550	6.00	1.007	95	9.8	88	33	107	42	4.3	16.3	1,670
8 D	2,770	6.03	1.006	102	8.7	93	36	109	46	4.7	17.0	1,745
9 D	2,730	6.07	1.006	97	8.4	98	34	114	44	4.9	16.8	1,740
10 D	2,480	6.15	1.005	88	6.7	94	30	107	43	4.6	16.3	1,736
11 D	2,940	6.28	1.006	100	6.6	97	27	111	43	6.0	16.6	1,639
12 D	2,510	6.22	1.006	95	5.9	93	28	109	40	5.5	15.3	1,807
13 D	2,435	6.29	1.007	87	5.1	98	28	103	44	5.7	15.6	1,778
14 D	2,570	6.27	1.007	85	5.4	94	29	99	44	6.4	16.2	1,773
15 D	2,825	6.30	1.006	103	4.7	93	29	112	47	7.1	16.8	1,730
16 D	2,670	6.30	1.006	80	4.5	89	29	96	44	6.0	16.1	1,675
17 D	2,860	6.33	1.006	92	4.5	95	27	109	44	7.6	16.7	1,745
18 D	3,100	6.42	1.005	104	4.4	95	23	117	43	9.3	16.1	1,682
19 D	2,850	6.40	1.006	111	4.4	97	26	126	46	8.3	16.7	1,746
20 D	2,630	6.45	1.006	81	4.2	92	22	94	42	7.7	15.1	1,710
21 D	2,570	6.45	1.007	88	4.2	92	23	97	43	7.6	15.8	1,709

* The daily average and the extremes of variation of the analyzed dietary components in the control period were: water, 3,800 ml.; Na (mean) 131, range, 129 to 133 mEq.; K, 108 mEq.; Cl, 232 mEq.; and N (mean) 16.9, range, 16.3 to 17.0 Gm. The figures for the depletion period were: water, 3,952, range 3,800 to 4,000 ml.; Na (mean) 124, range, 114 to 130 mEq.; K < 1 mEq.; Cl, 125 mEq.; and N (mean) 16.4, range, 16.1 to 16.7 Gm. In Tables I through IV where the daily range of variation is not given for the mineral constituents, the content in the milk of the diet was too small for accurate analysis; therefore, the amount of the substance added to the diet was virtually the total amount ingested. Where no variation is given for the fluid volume, the daily intake was constant for that period.

TABLE II

Metabolic data on Subject J. D. (normal Na^+ intake, Cl^- : HCO_3^- = 3 : 1)—Intake and urinary output of water, electrolytes, creatinine and nitrogen*

Day	Vol.	pH	S.G.	Urine Na	Urine K	Urine NH_4^+	Urine T.A.	Urine Cl	Urine N	Urine Cr
	ml.			*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*Gm.*	*mg.*
Control period										
1 C	2,400	5.78	1.010	123	82	46	28	158	16.8	2,196
2 C	2,770	6.28	1.011	142	118	43	24	205	20.0	2,126
3 C	2,500	5.87	1.010	112	86	43	29	133	20.1	2,156
4 C	2,390	6.07	1.010	93	112	38	25	134	19.3	2,103
5 C	2,810	6.55	1.009	165	100	34	13	179	19.0	2,158
Depletion period										
1 D	2,800	5.92	1.006	87	42	42	32	70	17.6	2,016
2 D	2,068	5.98	1.010	90	32	41	28	64	17.1	2,135
3 D	2,475	6.00	1.009	125	19	41	28	77	17.7	2,104
4 D	3,005	6.35	1.006	152	16	41	22	103	18.4	2,080
5 D	2,395	6.10	1.007	112	12	45	25	75	16.8	2,018
6 D	2,260	6.10	1.008	113	11	45	22	74	17.3	2,062
7 D	2,560	6.26	1.007	122	9	47	21	81	16.8	2,067
8 D	2,450	6.25	1.007	123	9	50	22	80	16.8	2,070
9 D	2,720	6.30	1.006	132	7.7	51	19	87	16.4	1,945
10 D	2,550	6.32	1.009	152	6.5	47	20	98	17.1	2,020
11 D	2,690	6.43	1.008	131	5.4	47	18	85	17.7	2,030
12 D	2,350	6.34	1.008	118	4.7	49	22	72	16.3	1,986

* The daily average and the extremes of variation of the analyzed dietary components in the control period were: water, 3,800 ml.; Na (mean) 128, range, 127 to 129 mEq.; K, 108 mEq.; Cl, 174 mEq.; HCO_3, 58 mEq.; and N (mean) 16.9, range, 16.9 to 17.0 Gm. The figures for the depletion period were: water, 3,800 ml.; Na (mean) 130, range, 129 to 132 mEq.; K < 1 mEq.; Cl, 94 mEq.; HCO_3, 31 mEq.; and N (mean) 16.8, range, 16.3 to 17.4 Gm.

per day intake, C. P.$_2$ and R. A. (Tables I and III), the nitrogen excretion decreased in relation to the nitrogen intake.

The excretion of *creatinine* showed no significant trend during the experiments.

The sum of the equivalents of *titratable acid*

TABLE III

Metabolic data on Subject R. A. (high Na^+ intake)—Intake and urinary output of water, electrolytes, creatinine and nitrogen*

Day	Vol.	pH	S.G.	Urine Na	Urine K	Urine NH_4^+	Urine T.A.	Urine Cl	Urine N	Urine Cr
	ml.			*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*Gm.*	*mg.*
Control period										
1 C	1,145	5.98	1.024	245	91	37	29	293	13.7	2,072
2 C	1,990	5.40	1.015	306	104	54	31	406	15.4	1,945
3 C	1,570	5.20	1.024	310	98	70	56	395	17.0	2,084
4 C	1,735	5.15	1.021	343	116	73	55	450	18.2	2,104
5 C	1,625	5.20	1.019	285	109	68	49	381	17.0	2,125
6 C	1,315	5.22	1.024	265	99	76	51	368	17.5	2,035
Depletion period										
1 D	1,420	5.40	1.019	224	63	71	47	291	15.5	1,860
2 D	1,875	5.55	1.019	235	38	74	47	269	16.1	1,973
3 D	1,615	5.62	1.015	235	28	80	44	258	17.5	2,249
4 D	1,645	5.61	1.013	233	20	65	31	255	14.1	1,968
5 D	1,940	5.63	1.023	191	17	61	33	203	13.6	1,828
6 D	1,360	5.90	1.021	268	17	74	38	274	16.1	2,360
7 D	2,205	6.21	1,012	321	11	60	25	339	13.0	1,725
8 D	1,525	6.03	1.014	232	12	61	28	236	12.9	1,731

* The daily average and the extremes of variation of the analyzed dietary components in the control period were: water (mean) 4,057, range, 3,840 to 4,250 ml.; Na (mean) 348, range, 347 to 349 mEq.; K, 110 mEq.; Cl, 454 mEq.; and Na (mean) 16.9, range, 16.5 to 16.9 Gm. The figures for the depletion period were: water (mean) 3,875, range, 3,800 to 4,050 ml.; Na (mean) 348, range, 347 to 349 mEq.; K < 1 mEq.; Cl, 345 mEq.; and N (mean) 16.3, range, 16.3 to 16.9 Gm.

and *ammonium ion* excreted by the kidney per 24 hours depended upon whether the subject had a portion of the total cations ingested with bicarbonate ions or almost entirely with chloride ions. In Subjects C. P.$_2$ and R. A., where the total cations ingested were associated almost entirely with chloride ions, the excretion of titratable acid plus ammonium ions was increased during the control periods and decreased during the potassium depletion periods (Tables I and III, Figure 3). In Subjects J. D. and J. B., where the total cations ingested were associated with bicarbonate and chloride ions in a ratio of 1:3, there was no consistent change in titratable acidity plus ammonium ion excretion during either the control or depletion periods (Tables II and IV, Figure 3). Both the titratable acid and ammonia increased in the urine during the control periods when bicarbonate was not added to the diet, Subjects C. P.$_2$ and R. A. (Figure 3). But in all four subjects the daily excretion of titratable acid decreased and urinary pH rose in the depletion period.

The urinary carbon dioxide and bicarbonate increased during the potassium depletion in Subject C. P.$_2$ (Table I).

Fecal excretion

These data from the subjects ingesting less than 1 mEq. potassium per day are presented in Table V.

The percentage of stool *solids* per stool collection period remained fairly constant for each individual except one (J. B.); there was, however, a wide variation in solids among the various subjects (8 to 34 per cent). The grams of *nitrogen* per 100 Gm. stool solids increased in three of these subjects and decreased in one. The amount

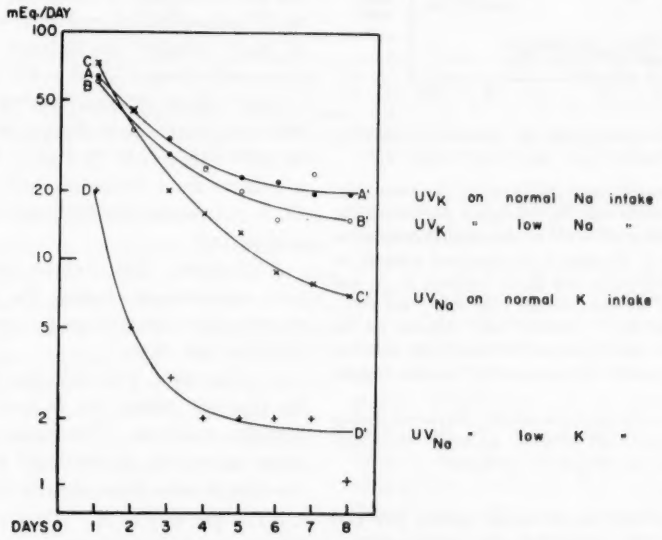

FIG. 2. THE RENAL CONSERVATION OF POTASSIUM AND SODIUM

The excretion rates of potassium are plotted against time for two subjects receiving 14 mEq. per day of potassium; one, E. H.$_2$, with a normal intake of sodium of 130 mEq. per day (curve A–A^1) and one, R. S.$_2$, with a low intake of sodium of 14 mEq. per day (curve B–B^1).

The excretion rates of sodium are plotted against time for two subjects receiving 14 mEq. per day of sodium; one, E. W.$_2$, with a normal intake of potassium of 90 mEq. per day (curve C–C^1) and one, R. S.$_2$, with a low intake of potassium of 14 mEq. per day (curve D–D^1).

This graph indicates that the rate of decline of urinary sodium was clearly more rapid than that of potassium under comparable reduction of intake of the respective ions. Sodium excretions were comparable on Day 0.

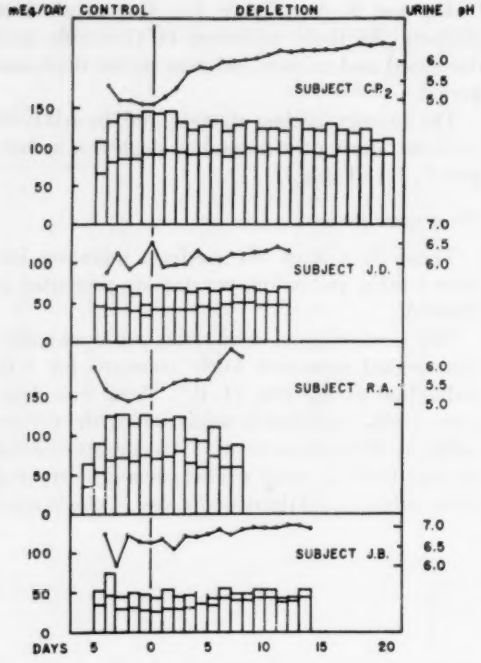

FIG. 3. THE DAILY EXCRETION OF AMMONIUM ION PLUS TITRATABLE ACIDITY AND THE DAILY URINE pH

Ammonium ion excretion is indicated by the lower portions and the titratable acid by the upper portions of the bars. The dots indicate the pH of the total 24 hour urine volume. Subjects C. P.$_2$ and J. D. received normal intakes of sodium (130 mEq. per day); Subjects R. A. and J. B. received high sodium intakes (340 mEq. per day). Subjects C. P.$_2$ and R. A. ingested only chloride as the supplemental anion and Subjects J. D. and J. B. received bicarbonate and chloride in a ratio of 1:3 as the supplemental anions.

Urinary pH rose and titratable acidity decreased during the depletion period in all subjects; excretion of ammonium ion was either unchanged or increased.

of *potassium* per unit stool solids (mEq. per 100 Gm.) decreased in every subject; nevertheless, the fecal loss of this ion accounted for from 11 to 59 per cent of the total loss in these subjects on the very low intake of potassium (Table VI). The amount of *sodium* per unit stool solids was increased in three of the four subjects and was unchanged in the other; this increase in stool sodium was not related to an increase in oral intake of the ion. There was no remarkable or consistent change in the fecal chloride excretion among the various subjects.

Total balances of electrolytes and nitrogen

These data are presented in Table VII.

1. Potassium. On normal intakes of sodium during the depletion period the negative balance of potassium tended to become greater the lower the level of potassium intake. When compared at the end of seven days of potassium depletion, Subject C. P.$_1$ lost 66 and Subject T. C. 165 mEq. of potassium while ingesting between 25 and 30 mEq. potassium per day; Subject E. H. ingesting approximately 14 mEq. of potassium per day lost 235 and 189 mEq. potassium during two separate studies; and at the lowest level of intake, less than 1 mEq. potassium per day, Subject C. P.$_2$ lost 260 and Subject J. D. lost 192 mEq. of potassium. The largest negative potassium balance, −502 mEq., was in one of the subjects in the last group, C. P.$_2$, who was maintained on less than 1 mEq. per day for three weeks. For the entire 26 days of the study in this patient the total potassium balance was −6.5 mEq. per Kg. of body weight; the balance of potassium "in excess of nitrogen" was −4.5 mEq. per Kg.

High intake of potassium in the control period led to a greater loss during the depletion period in both Subjects D. S. and L. P., but resulted in a greater final depletion only in L. P. since in D. S. potassium was retained initially in the control period.

2. Sodium. The sodium balances were positive consistently during the depletion periods except when the sodium intake was restricted to 14 mEq. per day.

3. Chloride. The chloride balance tended to be positive when the balance of sodium was strongly positive. The balance of chloride was most accurately determined in the subjects on the diet of resin-treated milk containing less than 1 mEq. per day of K. This was due to the fact that the resin treatment removed virtually all of the chloride: The amount of chloride put in the milk diet could be more accurately measured than that in the natural foodstuffs.

4. Nitrogen. Urinary nitrogen excretion for the most part reflected the nitrogen intake; however, in Subjects C. P. and R. A. (Tables I and III, respectively) the UV_N appeared to decline somewhat. This observation could not be accounted for by a decrease in nitrogen intake.

TABLE IV

Metabolic data on Subject J. B. (high Na^+ intake, Cl^- : HCO_3^- = 3 : 1)—Intake and urinary output of water, electrolytes, creatinine and nitrogen*

Day	Vol.	pH	S.G.	Urine Na	Urine K	Urine NH_4^+	Urine T.A.	Urine Cl	Urine N	Urine Cr
	ml.			*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*Gm.*	*mg.*
Control period										
1 C	3,850	6.80	1.007	263	77	35	16	263	15.6	1,940
2 C	1,420	5.98	1.018	233	44	47	29	231	15.6	2,130
3 C	2,840	6.78	1.012	355	95	30	16	337	18.6	2,002
4 C	2,610	6.60	1.012	326	75	31	19	298	16.5	2,010
5 C	2,600	6.58	1.013	365	121	28	20	360	17.0	2,048
Depletion period										
1 D	2,680	6.68	1.011	252	70	26	17	216	16.5	1,896
2 D	2,060	6.42	1.011	231	36	30	24	170	16.9	1,921
3 D	2,440	6.75	1.012	264	26	29	16	183	17.5	2,007
4 D	2,600	6.72	1.011	305	19	31	16	233	16.8	1,970
5 D	2,590	6.80	1.010	303	11	36		220	17.2	2,040
6 D	2,500	6.92	1.010	300	16	34	9	233	14.9	1,781
7 D	2,210	6.80	1.012	272	7.8	43	13	199	17.0	2,127
8 D	2,580	6.90	1.009	323	9.3	41	9	246	16.1	1,980
9 D	2,740	6.97	1.009	305	8.0	41	9	231	16.6	1,939
10 D	2,630	6.96	1.009	322	8.9	44	10	247	17.5	2,157
11 D	2,560	6.97	1.009	252	7.4	44	9	194	16.2	1,984
12 D	2,370	7.07	1.009	307	9.7	38	5	229	14.9	2,015
13 D	2,520	7.06	1.009	301	5.0	39	5	209	16.4	1,909
14 D	2,760	6.94	1.009	360	5.8	45	9	277	16.1	1,980

* The daily average and the extremes of variation of the analyzed dietary components for the control period were: fluid volume (mean) 3,837, range, 3,699 to 4,000 ml.; Na (mean) 312, range, 168 to 348 mEq. (Na^+ intake increased stepwise during control period); K (mean) 98, range, 55 to 109 mEq. (K^+ intake too low on the first day due to dietary error); Cl (mean) 303, range, 161 to 339 mEq.; HCO_3 (mean) 101, range, 54 to 113 mEq.; and N (mean) 16.8, range, 16.3 to 17.2 Gm. The figures for the depletion period were: fluid volume 3,800 ml.; Na (mean) 348, range, 347 to 349 mEq.; K < 1 mEq.; Cl, 259 mEq.; HCO_3, 86 mEq.; and N (mean) 16.5, range, 15.6 to 17.3 Gm.

Concentrations of electrolytes in serum

The concentration of potassium in serum fell progressively during the depletion period in all experiments, reaching the lowest value of 2.8 mEq. per L. in the subject on the lowest intake of K for the longest period, C. $P._2$ (Table VIII). There were no apparent trends in the daily serum concentrations of sodium, chloride or creatinine. The venous CO_2 content rose slightly in Subjects C. $P._2$ and R. A. but remained within normal limits.

Acid-base changes in "arterialized" finger capillary blood

Periodic samples of "arterialized" finger capillary blood showed no major changes in the whole blood pH, CO_2 content or "buffer base" (Table VIII).

DISCUSSION

Our data indicate that subjects ingesting an adequate diet containing at least 25 mEq. of potassium per day are unlikely to develop potassium depletion greater than 250 mEq. as the result of urinary potassium loss alone. This is probably also true for intakes of 14 mEq. per day. At both levels of intake the urinary potassium daily loss had reached or almost reached the intake levels within the duration of these studies (Figure 1). At the extremely low intake of 1 mEq. per day, however, the decline in the urinary loss was very slow in the last five days of a 21 day study (C. $P._2$) as shown in Figure 4. Therefore, under the circumstance of prolonged and practically complete deprivation of potassium, the urinary loss, while not more than about 3 mEq. above the intake, could reasonably be expected to continue to add significantly to the deficit of the ion.

In the first day of depletion, however, the drop in potassium intake is a more significant factor in determining the contribution of the urinary potassium loss to the depletion. Those subjects loaded with potassium during the control period,

TABLE V

Analysis of average daily stool values

Subject	Stool collection: Period	Stool collection: Duration	Total weight	Total solids	Na	K	Cl	N	K/Na ratio
		days	*Gm.*	*Gm.*	*mEq.*	*mEq.*	*mEq.*	*Gm.*	
C. P.$_2$	Control period								
	I	2	525	71.0	13.2	40.3	4.9	3.6	3.1
	II	3	175	30.1	2.6	22.2	1.8	1.4	8.5
	Depletion period								
	I	3	193	36.5	4.3	24.9	1.8	1.6	5.8
	II	3	155	27.6	7.2	15.9	1.5	1.8	2.2
	III	3	207	38.3	13.4	16.3	2.1	1.8	1.2
	IV	3	173	30.2	9.0	14.1	1.5	1.6	1.6
	V	3	168	31.4	7.8	12.0	1.4	1.6	1.5
	VI	3	174	29.8	8.0	9.5	1.2	1.5	1.2
	VII	3	164	31.9	7.6	11.4	1.0	1.7	1.5
J. D.	Control period								
	I	2	103	31	0.9	15.5	0.5	1.05	17.2
	II	3	77	22	0.7	12.5	0.3	0.70	17.9
	Depletion period								
	I	3	52	16	1.1	8.1	0.3	0.49	7.4
	II	3	66	21	3.0	9.2	0.3	0.63	3.1
	III	3	60	18	4.0	5.7	0.5	0.59	1.4
R. A	Control period								
	I	4	106	8.2	7.0	8.8	2.5	1.15	1.26
	II	2	110	8.8	4.5	14.0	5.0	1.10	3.11
	Depletion period								
	I	6	53	5.0	2.5	3.3	0.2	0.80	1.32
	II	2	52	3.6	2.5	2.5	0.5	0.70	1.00
J. B.	Control period								
	I	2	117	17	2.5	18.2	1.4	0.80	7.3
	II	3	68	29	1.7	7.8	0.6	0.38	4.6
	Depletion period								
	I	3	72	26	4.7	8.6	0.6	0.60	1.8
	II	3	40	13	2.8	4.0	4.0	0.35	1.4
	III	2	79	26	7.4	5.8	5.8	0.75	0.78
	IV	2	44	17	4.7	3.8	3.8	0.55	0.81
	V	4	39	12	3.1	2.6	3.8	0.50	0.84

D. S. and L. P., lost, respectively, 129 and 127 mEq. in the urine on the first depletion day as contrasted with losses of 29 to 52 mEq. in the other subjects on a comparable depletion intake (25 mEq. per day). However, despite the greater urinary loss on the first day in these subjects

TABLE VI

Per cent of total potassium loss and per cent solids in stools during potassium depletion period

Subject	Period duration	Urine K	Stool K	Total K loss	K loss in stool	Stool solids
	days	*mEq.*	*mEq.*	*mEq.*	%	%
Normal sodium intake						
C. P.$_2$	21	216	307	523	59	19
J. D.	12	174	86	260	33	31
High sodium intake						
R. A.	8	206	25	231	11	8
J. B.	14	270	67	307	22	34

TABLE VII

Summary of balance data

Subject	Control period: Period duration	Control period: Change in wt.	Control period: Balance K	Control period: Balance Na	Control period: Balance Cl	Control period: Balance N	Depletion period: Period duration	Depletion period: Change in wt.	Depletion period: Final wt.	Depletion period: Total period balance K	Depletion period: Total period balance K*	Depletion period: Total period balance Na	Depletion period: Total period balance Cl	Depletion period: Total period balance N	7th depletion day values: Cumulative UV_E	7th depletion day values: Cumulative balance K	7th depletion day values: Cumulative balance K*
	days	*Kg.*	*mEq.*	*mEq.*	*mEq.*	*Gm.*	*days*	*Kg.*	*Kg.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*Gm.*	*mEq.*	*mEq.*	*mEq.*
<1 mEq. K/day depletion diets																	
Normal Na intake																	
C. P.$_2$	5	−1.31	−79	− 55	− 17	−19.8	21	−2.75	76.8	−502	−399	+889	+408	−36.8	128	−260	−226
J. D. (HCO_3)†	5	−1.40	−27	+ 2	+ 59	−14.7	12	−2.68	85.8	−248	−218	+ 67	+157	−10.8	143	−192	−175
Oral Na loading in control and depletion periods (350 mEq./day)																	
R. A.	6	−1.81	−21	+298	+411	− 9.2	8	−0.85	88.6	−225	−244	+825	+660	+ 6.7	188	−207	−223
J. B. (HCO_3)†	4	−1.02	+16	+ 12	− 57	− 1.3	14	−0.60	84.3	−293	−278	+716	+553	− 5.3	235	−235	−229
14 to 16 mEq. K/day depletion diets																	
Normal NaCl intake																	
E. H.$_1$	3	−1.0	−31	− 93	−167	±	8	−0.50	70.4	−251	−217	+232	+102	−12.2	305	−235	−205
E. H.$_2$	4	+0.1	−69	− 64	−104	− 2.0	8	−1.20	70.0	−206	−196	+202	+ 69	− 3.7	232	−189	−180
Low NaCl intake during depletion period																	
R. S.$_1$	3	−1.21	+44	− 84	−171	+ 2.9	7	−1.78	73.0	−176	−100	− 12	−100	−27.2	258	−176	−100
R. S.$_2$	4	−0.14	−96	+ 12	− 14	− 0.3	8	−1.19	72.5	−183	−153	− 57	−123	−10.7	210	−167	−141
25–27 mEq. K/day depletion diets																	
Normal Na intake																	
T. C.	3	−2.1	−38	+ 18	− 93	− 6.0	8	−0.70	85.9	−172	−162	+130	− 17	− 3.4	138	−165	−155
C. P.$_1$	6	−1.3	−74	+103	+ 68	−12.8	7	−0.80	74.3	− 66	− 46	+195	+215	− 7.1	263	− 66	− 46
Oral K loading in control period (300 mEq. K/day)																	
D. S.	7	−0.3	+79	+ 90	− 11	+14.3	7	±	67.7	−208	−253	+ 44	− 74	+16.2	275	−208	−253
L. P.	7	−0.9	+ 8	+ 19	− 85	− 4.6	6	−1.00	69.1	−244	−238	+289	+ 74	− 2.2			
Oral NaCl loading in control and depletion periods (340 mEq. Na/day)																	
E. W.$_1$	7	±	−25	+ 37	− 71	+13.7	8	+0.30	67.5	−158	−197	+296	+323	+13.8	240	−146	−180
H. F.	7	−2.1	−53	+116	− 87	−13.2	8	−0.60	84.2	− 79	− 60	+475	+324	− 6.7	256	− 97	− 81

* K^1 is potassium balance corrected for the nitrogen balance, assuming that 1 Gm. N = 2.8 mEq. K.

† Cl : HCO_3 intake ratio = 3 : 1.

TABLE VIII

Analysis of blood and serum

Subject	Day	Whole blood*						Venous serum				
		pH	CO_2	pCO_2	Bb	Cell vol.	Hb	Na	K	Cl	CO_2	Creatinine
			mMoles/L.	*mm. Hg*	*mEq./L.*	%	*mMoles/L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mMoles/L.*	*mg. %*
C. P.$_2$	C 5	7.39	21.7	42.0	48.5	46.5	9.20	140	4.1	104	26.1	1.10
	D 7	7.41	20.5	38.0	48.0	46.5	9.15	140	3.5	104	26.9	0.93
	D 12	7.42	20.6	38.0	48.0	44.0	9.00	140	3.3	104	28.0	1.20
	D 14	7.44	21.5	38.0	49.5	44.5	9.00	140	3.1	106	28.6	1.60
	D 16	7.41	21.1	39.0	48.0	43.0	8.65	140	3.1	104	30.6	1.10
	D 19	7.43	21.1	38.0	48.5	43.5	8.85	140	2.8	104	29.8	1.40
	D 21	7.43	21.1	38.0	49.0	44.0	8.95	141	2.8	107	29.6	0.90
J. D.	C 5	7.44	21.3	38.0	50.0	47.0	9.55	140	4.0	104	29.3	1.10
	D 6	7.42	21.0	38.0	48.5	45.5	9.25	140	3.9	102	28.8	0.93
	D 12	7.40	21.0	40.5	48.5	48.0	9.60	140	3.7	103	29.8	0.85
R. A.	C 3	7.38	18.4	37.0	45.5	47.0		138	4.0	105	23.1	1.20
	D 8	7.45	19.8	34.0	48.0	45.0		143	3.0	104	30.1	0.92
J. B.	C 5	7.44	22.1	38.5	50.0	43.0	8.65	139	4.2	105	31.2	0.90
	D 4	7.45	21.3	36.5	49.0	41.5	8.25	140	4.0	104	30.9	1.00
	D 8	7.43	20.9	36.5	47.5	40.0	7.85	140	3.5	104	31.0	1.70
	D 14	7.43	21.8	38.0	47.5	38.5	7.70	140	3.6	103	30.5	0.93

* Arterialized cutaneous whole blood (16). Normal values: pH 7.42 ± 0.04; CO_2 21.2 ± 2.6 mMoles per L.: BB (buffer base) 48.8 ± 3.8 mEq. per L.; pCO_2 37.6 ± 7.2 mm. Hg.

loaded with potassium in the control period, their urinary losses declined very rapidly. This impetus to renal conservation of potassium (the difference between control and depletion intakes) in the first day of depletion is shown clearly in Figure 5 where the decrement in intake corre-

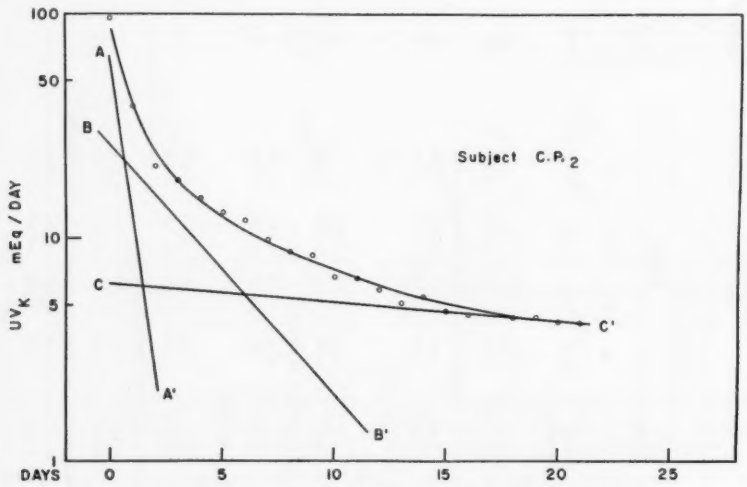

FIG. 4. THE RATES OF RENAL POTASSIUM EXCRETION DURING POTASSIUM DEPLETION

At least three exponential rates may be derived from the curve describing the daily urinary potassium excretion during potassium depletion (Subject C. P.$_2$): A–A′ = (64) ($e^{-1.69d}$), B–B′ = (26) ($e^{-0.79d}$) and C–C′ = (6.3) ($e^{-0.019d}$). For further discussion, see text.

lates well with the estimated instantaneous rate of decline in urinary potassium at the end of the first day. This correlation becomes less certain at the end of the fourth depletion day and probably disappears by the end of the seventh day.

The decline in the urinary potassium content in the first three days of depletion was roughly comparable to that observed by Black and Milne (2) on similar potassium intakes.[3] However, the curve for urinary potassium could not be described by a single exponential function, as in their study. An analysis of the curve for the subject depleted for the longest period, C. P.$_2$, 21 days, showed that it could be reasonably well described as the sum of three exponential functions (Figure 4). This analysis does not necessarily imply the identification of three physiological functions in the body which determine urinary potassium content under the circumstances of the study, but it does serve as a convenient way to describe the shape of the curve.

The rate of renal potassium conservation was slower than that for sodium, as previously described (2). The improvement in potassium conservation during a simultaneous reduction in sodium intake is certainly small, if significant at all (Figure 2, curves A–A′ and B–B′). Sodium, however, appears to be conserved much more effectively during a simultaneous reduction in potassium intake (Figure 2, curves C–C′ and D–D′). These curves emphasize the greater effectiveness of renal conservation of sodium as compared to that for potassium.

No other attempts were made to study changes in renal function which might follow potassium depletion. Whatever impairment of concentrating power and resulting polyuria might have been found as described so well in rat studies by Hollander and associates (18) was obscured by the large fluid intakes required by the study

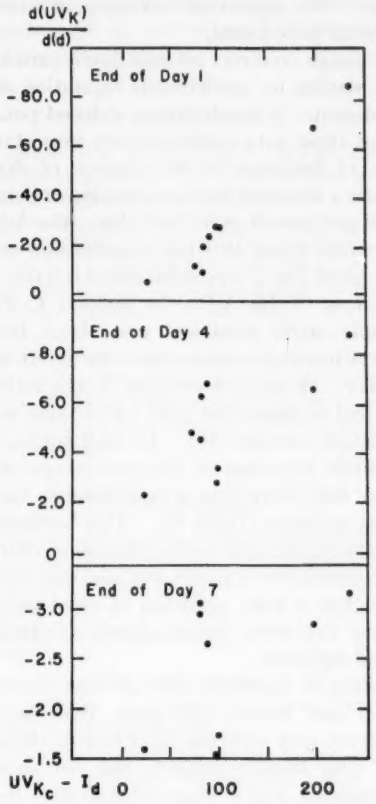

FIG. 5. THE DAILY RATE OF POTASSIUM EXCRETION DURING THE POTASSIUM DEPLETION PERIOD

The instantaneous rate of decline of urinary potassium excretion, $\frac{d\,(UV_K)}{d\,(days)}$, is plotted as a function of the average of the urinary potassium excretion for the last three days of the control period, UV_{KC}, minus the potassium intake during the depletion period, I_d. The quantity $UV_{KC} - I_d$ is taken as an index of the change in potassium load between the control and the depletion periods. The differential $\frac{d\,(UV_K)}{d\,(days)}$ was derived by differentiation of exponential curves fitted to the data of daily urinary potassium loss as in Figure 4. Any correlation existing between these two variables becomes less apparent the farther along in the depletion period the observation is made.

[3] Black and Milne (2) measured the rate at which potassium conservation becomes effective by evaluating α in the following expression:

$$Q_t = Q_0 \times e^{-\alpha t}$$

in which Q_t is the potassium output on day t, and Q_0 is the initial potassium output. This equation for the data of Black and Milne is:

$$Q_t = 106 \times e^{-0.805t}$$

This equation for Subjects J. B. and R. A. is:

$$Q_t = 103 \times e^{-0.414t}$$

The value of α obtained for our subjects indicates a more rapid rate of potassium conservation compared to those of Black and Milne. This difference may not be significant. The αK of 0.414 obtained for our studies corresponds more closely to the αNa of 0.44 obtained by Black and Milne from a sodium conservation study (2).

design. No significant changes in creatinine clearance were found.

Although no direct information is provided by these studies on mechanisms regulating the loss of potassium in stools during reduced potassium intake, these data emphasize how important this route of loss was in the absence of diarrhea. Despite a tendency for conservation of potassium in the gut as well as in the kidney, the daily loss of between 2 and 25 mEq. of potassium in stools made stool loss a very substantial fraction of the total loss (Table VI). In Subject C. P.$_2$, for example, daily stool loss was about 10 mEq. when urinary potassium was only about 5 mEq. per day. A marked increase in the fluidity of the stool is associated with an increase in stool potassium content (19). In each subject there was little variation in the percentage of stool solids, but there was a considerable variation among subjects (Table V). This variation was not related, though, to the potassium content of these nondiarrheal stools and one can only conclude that a wide variation of stool potassium content can exist independently of fluidity in normal subjects.

Studies of Gardner, MacLachlan, Terry, McArthur and Butler (20) show that potassium deficiency may increase fecal loss of chloride in rats. Our human subjects did not show this phenomenon, but it must be conceded that the degree of their depletions were much smaller relative to body weight than were those of the rats. Perhaps the most surprising finding was the very modest degree of metabolic alkalosis which developed in any of these studies. The subject, C. P.$_2$, with the largest potassium deficit, 502 mEq. (351 mEq. when corrected for nitrogen balance), showed a rise in venous serum CO_2 content of only 3.5 mMoles per L. from a control level of 26.1 during his depletion period of 21 days. His arterial whole blood pH rose from 7.39 to only 7.43. Because in a previous study of experimental potassium depletion in man (2) more striking rises in serum CO_2 were noted when sodium loading accompanied the depletion (rises to 36.1 and 33.4 mEq. per L.), two of our subjects were given sodium loads of 350 mEq. per day. With potassium depletions comparable in degree to those of Black and Milne (2), one of the subjects (R. A.) developed a comparable rise in serum CO_2 (+7.0 mEq. per L.) but at a lower level (final CO_2, 30.1). The second subject (J. B.) showed not even a tendency toward metabolic alkalosis. The authors conclude that human subjects must show a great variability in susceptibility to metabolic alkalosis in the presence of a pure potassium depletion and agree with Moore and co-workers (21) that sodium loads must play a very significant part in the appearance of the alkalosis. A subsequent paper discusses the acid-base problem in experimental potassium depletion in greater detail (22).

A final comment must be made on the failure of the arterialized cutaneous blood total CO_2 values to parallel the venous serum CO_2 in Subjects C. P.$_2$ and R. A. (Table VIII). The authors do not believe that technical errors account for this failure but the only explanation they can offer is a speculative one. The red cell, in potassium depletion, may show a replacement of potassium lost by a sodium gain (23) like that shown by skeletal muscle (24). Gardner, MacLachlan and Berman have shown that this change in skeletal muscle composition is accompanied by a decrease in bicarbonate content (25). If a like change in red cell bicarbonate took place in the subjects under discussion, these decreases could have cancelled out or masked the rises in serum CO_2, inasmuch as the cutaneous blood analyses were done on whole blood.

SUMMARY AND CONCLUSIONS

Fourteen balance studies were done on 11 normal male subjects depleted of potassium by dietary deprivation of the ion. The basic study plan consisted of a control period lasting three to eight days and a succeeding depletion period lasting 6 to 21 days during which the potassium intake was set at one of three levels: 25 to 27, 14 to 16 or <1 mEq. per day. During the depletion period the effects of the following diet programs on the urinary and fecal potassium loss were observed: *1*) ingestion of a greatly augmented sodium intake through the entire study; *2*) ingestion of sodium and potassium entirely with chloride or with chloride and bicarbonate in a ratio of 3:1; *3*) ingestion of a low intake of sodium of 14 mEq. per day during the control period; and *4*) ingestion of a. high intake of

potassium in excess of 300 mEq. per day during the control period.

The rate of *urinary excretion of potassium: 1*) during the first few days of depletion varied directly with the difference between the urinary potassium of the control period and the potassium intake in the depletion period; *2*) varied directly with the level of potassium intake throughout the depletion period; but *3*) clearly exceeded the intake when the latter was 1 mEq. or less per day; *4*) declined at two or more exponential rates when the potassium intake was <1 mEq. per day; *5*) was increased by sodium loading at the lowest level of potassium intake; and *6*) was not affected by the association of chloride alone *versus* chloride plus bicarbonate with the total cation ingested.

Fecal potassium accounted for 11 to 59 per cent of the total loss of potassium when the intake of potassium was less than 1 mEq. per day. Cumulative negative *balances* of potassium varied with level of intake and duration of study reaching a maximum of −502 mEq. or 6.4 mEq. per Kg. in one subject on less than 1 mEq. per day for 21 days.

The rate of *urinary excretion of sodium* on a low sodium intake was diminished when the concomitant potassium intake was low. The rate of excretion of *titratable acid plus ammonium ion* remained essentially unchanged or decreased slightly during potassium depletion and the urine pH rose. *Urinary total CO_2 and bicarbonate* increased in the one subject in which it was measured and the calculated total excretion of hydrogen diminished relative to dietary intake.

Metabolic alkalosis was an inconstant finding and when present was of a minor degree.

It is concluded: *1*) that the degree of renal conservation of potassium *a*) is related to the degree of change in intake of the ion from predeprivation levels and to the duration of the deprivation, *b*) is diminished by an increased intake of sodium, and *c*) is less efficient than the renal conservation of sodium under conditions of comparable deprivation of the respective ions; *2*) that with prolonged deprivation of potassium the fecal loss of the ion, though absolutely decreasing, becomes a progressively larger factor in the development of the negative potassium balance; and *3*) that a marked degree of extracellular metabolic alkalosis is not an obligatory accompaniment to potassium depletion of the normal human subject.

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EXPERIMENTAL POTASSIUM DEPLETION IN NORMAL HUMAN SUBJECTS. II. RENAL AND HORMONAL FACTORS IN THE DEVELOPMENT OF EXTRACELLULAR ALKALOSIS DURING DEPLETION*

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In the preceding paper (1) experimental depletion of potassium in normal human subjects did not produce an extracellular metabolic alkalosis of the severity which usually occurs in patients with comparable potassium deficits. Much investigative study has been applied to this association but, because of the many factors involved in such patients, the relative importance of such factors in the etiology of this association in man remains unclear.

Most of our knowledge of the metabolic derangements induced in mammals by potassium depletion has come from studies in rats (2–12). The depletion in rats is usually accompanied by metabolic alkalosis of varying severity. A hypothesis accounting for the alkalosis has been formulated by Darrow, Cooke and coworkers (2–4) and has been given additional support by the studies of Orloff, Kennedy and Berliner (9). In brief, the loss of potassium from cells is replaced in part by hydrogen ion from extracellular fluid, the latter shift resulting in an intracellular acidosis and an extracellular alkalosis. This primary cellular distortion of acid-base equilibrium is then maintained by the kidney by an enhancement of tubular reabsorption of bicarbonate over that of chloride and the excretion of a relatively acid urine. In these rat studies the absence or presence and degree of alkalosis appear to be related to many variables including the duration and severity of the depletion, the protein content of the diet, the acid-base composition of the diet and the use of desoxycorticosterone in promoting depletion.

Evidence has been reported that this phenomenon can be induced experimentally in man (13, 14). But potassium depletion in man does not always result in an unequivocal extracellular alkalosis either experimentally (1) or clinically (15), and Moore and co-workers (16) have shown that loss of acid as gastric fluid and interference by adrenocortical hormones with renal compensations for internal exchanges may be at least as important in causing a manifest alkalosis.

The experiments in this paper were devised to provoke in normal man the appearance of extracellular metabolic alkalosis in conjunction with potassium depletion, without and with concomitant administration of desoxycorticosterone, and without variation of the anion-cation balance of the intake high in sodium or loss of chloride in gastric fluid. The analysis of the results has been aimed at examining the validity of the Darrow hypothesis for man. The derived data confirm an internal shift of hydrogen from extracellular fluid and show little alteration of extracellular acid-base equilibrium by changes in the renal excretion of acid, which changes appear to influence primarily the intracellular content of acid.

EXPERIMENTAL PROCEDURE AND METHODS

Human subjects and design of study. Four metabolic balance studies were done on three volunteer medical students in good health. They gave no history of any past illness that might affect the studies. The basic study design was that of a five day control period followed by a potassium depletion period of 12 to 16 days. The potassium depletion period proceeded for seven to 10 days and then desoxycorticosterone acetate (DCA) (20 mg. per day in oil given intramuscularly) was added to the program for five to six days. In all the studies, chloride (Cl^-) and bicarbonate (HCO_3^-) were consumed in a ratio of 3:1; one subject, J. M.$_1$, received 129 mEq. of sodium per day throughout, the other subjects received 348 mEq. per day.

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† Established Investigator of the American Heart Association.

During depletion, the potassium intake averaged about 1 mEq. per day.

The specific studies were designed as follows: The control period for each subject was five days; the depletion period was seven days for J. M.$_2$, eight days for J. M.$_1$ and R. H., and 10 days for J. S.; the succeeding period of depletion plus DCA was five days for J. M.$_1$ and J. M.$_2$, and six days for R. H. and J. S.; in addition R. H. was further depleted after DCA for two days and then repleted for two days.

TABLE I

Venous serum and arterialized cutaneous blood values

Subject	Day	Venous serum				Arterialized cutaneous blood					
		K	Na	Cl	CO_2	pH	CO_2	pCO_2	BB*	Hct.	Hb.
		mEq./L.	*mEq./L.*	*mEq./L.*	*mMoles/L.*		*mMoles/L.*	*mm. Hg*	*mEq./L.*	%	*mMoles/L*
Normal Na^+ intake											
J. M.$_1$											
Initial											
	3					7.43	22.8	41	50	44	9.6
	5	4.0	138	104	28.7						
Depletion											
	1	4.0	138	110	27.4						
	2	4.0	137	105	26.4						
	3	3.8	138	107	27.1	7.38	21.5	42	47	44	7.5
	4	3.7	137	105	27.5						
	5	3.8	138	108	24.6						
	6	3.7	138	107	28.3						
	7	3.8	138	104	28.2						
	8	3.3	138	104	28.0	7.44	23.3	40	50	40	8.6
Depletion + DCA											
	9	3.9	138	101	29.1	7.41	22.5	41	48	43	8.6
	10	3.3	138	103	31.1	7.38	23.2	45	48	42	8.2
	11	3.3	138	103	28.7	7.41	22.4	41	48	40	8.4
	12					7.43	22.9	40	49	40	8.4
	13	3.1	138	94	29.3	7.38	23.1	45	48	39	8.1
High Na^+ intake											
J. M.$_2$											
Initial											
	5	4.0	141	105	24.5	7.39	20.7	40	47	44	8.7
Depletion											
	1	4.0	140	105	26.0						
	2	3.8	140	105	26.0	7.39	20.8	40	47	45	8.5
	3	3.5	141	106	24.7						
	4	3.5	140	108	25.3	7.39	20.1	40	46	46	9.0
	5	3.6	140	107	24.8						
	6	3.5	140	107	25.7	7.42	21.0	38	47	40	8.5
	7	3.5	141	107	26.4	7.42	21.6	39	48	40	8.2
Depletion + DCA											
	8	3.3	140	106	27.1	7.44	22.6	39	49	39	7.9
	9	3.1	140	106	27.8	7.46	23.5	39	50	37	8.0
	10	2.8	143	106	25.8	7.43	22.4	39	50	41	8.1
	11	2.8	143	106	27.9		22.8			37	
	12	2.7	143	106	28.1	7.45	22.1	36	48	35	7.5
R. H.											
Initial											
	5	4.0	145	104	30.3	7.40	22.5	44	51	53	9.5
Depletion											
	1	4.0	145	103	30.0						
	2	3.9	144	104	28.0	7.43	21.8	40	50	48	9.4
	3	3.6	144	105	26.9						
	4	3.5	143	106	28.2	7.44	21.8	39	50	46	9.1
	5	3.5	145	107	29.4						
	6	3.7	148	106	27.6	7.46	22.4	39	51	48	9.3
	7	3.5	147	105	28.4	7.42	21.2	40	49	47	9.1
	8	3.4	145	106	29.1	7.43	22.7	41	51	46	9.0

TABLE I—*Continued*

Subject	Day	Venous serum				Arterialized cutaneous blood					
		K	Na	Cl	CO_2	pH	CO_2	pCO_2	BB*	Hct.	Hb.
		mEq./L.	*mEq./L.*	*mEq./L.*	*mMoles/L.*		*mMoles/L.*	*mm. Hg*	*mEq./L.*	%	*mMoles/L.*
Depletion + DCA											
	9	3.4	146	105	30.5	7.43	22.6	41	51	47	9.3
	10	2.8	147	105		7.45	23.3	41	52	45	9.1
	11	2.9	146	105	29.7	7.45	23.0	39	50	42	8.5
	12	3.0	147	107	29.9	7.45	24.0	42	52	43	8.2
	13	3.0	147	105	30.3	7.46	23.9	40	52	44	8.5
	14	3.0	147	105	30.7	7.46	24.4	42	53	43	8.7
Depletion											
	15	3.1			28.4	7.44	24.2	42	51	41	7.9
	16	3.1	146	107	29.9	7.43	23.9	42	51	41	8.0
Repletion											
	1	3.7	143	107	27.4	7.42	21.4	49	48	42	8.3
	2	4.2	146	105	28.3	7.43	21.2	38	49	43	8.8
J. S. Initial											
	5	4.0	140	97	28.1	7.38	21.5	44	48	47	9.1
Depletion											
	1	4.0	140	97	32.1						
	2	3.6	140	102	31.1						
	3	3.9	140	103	34.1						
	4	3.9	139	101	32.6						
	5	3.4	140	101	33.0	7.43	24.7	41	50	43	8.5
	6	3.1	140	99	28.5	7.46	24.7	41	52	43	8.6
	7	3.3	140	101	28.5						
	8	3.3	140	99	33.2						
	9	3.1	140	104	33.9						
	10	3.2	140	103	36.6	7.43	24.7	41	53	44	8.8
Depletion + DCA											
	11	3.0	140	100	34.1						
	12	3.1	140	97	37.6	7.45	25.4	45	54	43	8.7
	13	3.0	140	104	35.1	7.44	26.3	46	53	40	8.0
	14	3.0	140	100	35.1	7.46	28.2	47	56	41	8.0
	15	2.8	140	96	33.5	7.45	29.4	50	57	44	8.9
	16	2.7	140	93	36.0	7.47	30.1	49	58	41	8.1

* Buffer base.

The subjects slept, took meals and gave metabolic specimens on the Metabolic Unit. They were allowed normal daytime activities; during studies in warm months, subjects were confined largely to the Metabolic Unit or air-conditioned parts of the hospital. Specimens were collected as in the previous study (1).

Diets. The subjects ingested prepared milk and vitamin supplements. The milk was rendered practically free of monovalent ions by repeated passage through mono-bed cation and anion ion exchange resins.[1] These resins yield hydroxyl ion for monovalent anions and hydrogen ion for cations. The milk was then reconstituted with weighed portions of sodium and potassium chloride and bicarbonate as required by the study plan. The amount of milk ingested each day was held constant; the protein and caloric intake approximated normal levels. No additional water was drunk by the subjects. Milk was treated, as described above, every two days, and an aliquot from each lot was analyzed for sodium, potassium, chloride, nitrogen and solids content. Several aliquots were analyzed for calcium and phosphorus and were found to contain about two-thirds of the amounts in normal milk.

Chemical methods and calculations. The milk and metabolic specimens (urine, stool and blood) were analyzed by the chemical methods presented in the preceding paper (1). Metabolic calculations were carried out as described in detail elsewhere (17) and as summarized in part below.

[1] Amberlite MB-3, Rohm and Haas, Philadelphia, Pa.

RESULTS: OBSERVED

The data are presented in Tables I to III and in Figures 1 to 7.

Blood and serum changes

The major changes found were in the serum potassium level and in the indicators of acid-base equilibrium (Table I, Figure 1).

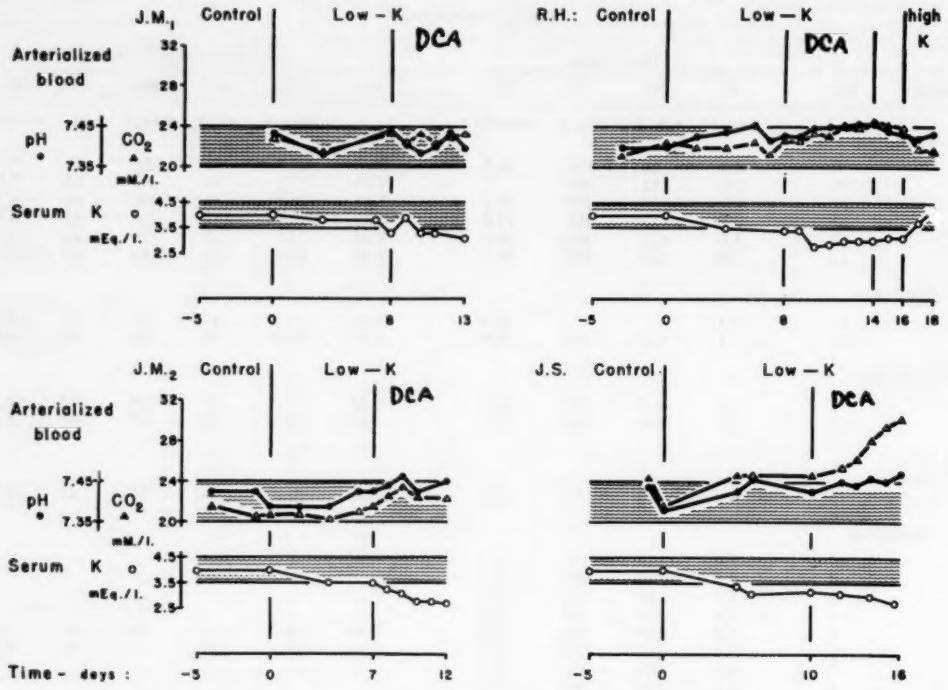

FIG 1. TOTAL CO_2 CONTENT AND pH OF ARTERIAL BLOOD AND VENOUS SERUM CONCENTRATION OF POTASSIUM DURING POTASSIUM DEPRIVATION AND ADMINISTRATION OF DESOXYCORTICOSTERONE

Serum potassium concentration fell below normal in all four subjects. The arterial CO_2 tended to rise slightly in two and only rose definitely to alkalotic levels in one of the four subjects.

All of the subjects started the depletion period with serum potassium concentrations of 4.0 mEq. per L. As depletion progressed, the concentrations fell progressively with the greatest decline being to 2.7 mEq. per L. in 12 days and the smallest to 3.1 mEq. per L. in 13 days.

During depletion, *acid-base variables* changed at some time in the three subjects on a high sodium intake in the direction of metabolic alkalosis when compared with the final values of the predepletion period. For the venous serum CO_2 content, the maximum rises ranged from +0.4 to +9.5 mMoles per L. The maximum rises in cutaneous arterialized whole blood variables ranged as follows: CO_2 content, +0.5 to +8.6 mMoles per L.; pH, +0.01 to +0.09 units; and buffer base, 0 to +10 mEq. per L. The largest changes took place during the period of DCA administration. Respiratory compensation was fairly good as shown by a rise in calculated pCO_2 and by a maximum pH of 7.47.

External balances

Urine *potassium* declined rapidly to an average output on the seventh day of depletion of 12 mEq. in the subjects on high sodium intakes (J. $M._2$, R. H. and J. S.) and to 7 mEq. in the subject on normal sodium intake (J. $M._1$) (Table II). Stool content of potassium also decreased during depletion.

DCA raised urinary potassium to daily maxima of nine to 25 mEq. above the output of the last day of depletion without DCA. The stool content of potassium increased similarly to a lesser degree.

The total negative balance of potassium attained by the end of the depletion, 12 to 16 days (including the DCA period), ranged from −288 to −618 mEq. (−221 to −502 mEq. when

TABLE II

Urine and stool analyses

Subject	Day	Urine											Stool§			
		Vol.	K^+	Na^+	Cl^-	N	NH_4^+	T.A.*	HCO_3^-	H^+†	PO_4‡	pH	K^+	Na^+	N	Solids
		ml.	*mEq.*	*mEq.*	*mEq.*	*Gm.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*		*mEq.*	*mEq.*	*Gm.*	%
Normal Na^+ (*ca.* 130 mEq. per day)																
J. M.$_1$ Initial	1	2,505	79	158	182	12.6	31	12	21	22	37	6.77	18	3.3	1.28	29
	2	2,290	93	89	154	15.5	42	17	5	54	26	6.22	18	3.3	1.28	29
	3	2,495	113	89	146	17.2	49	18	6	61	32	6.35	18	3.3	1.28	29
	4	2,430	92	119	153	17.2	47	21	8	60	33	6.31	18	3.3	1.28	29
	5	2,430	92	119	153	17.2	47	21	8	60	33	6.31	18	3.3	1.28	29
Depletion (*ca.* 1 mEq. per day)																
	1	2,650	40	101	93	16.7	45	24	5	64	35	6.20	9.2	4.9	0.65	25
	2	2,060	22	87	72	16.5	50	25	2	73	35	6.10	9.2	4.9	0.65	25
	3	2,650	17	109	78	16.7	48	27	6	69	45	6.30	9.2	4.9	0.65	25
	4	2,480	13	104	78	16.8	48	28	3	73	46	6.25	9.2	4.9	0.65	25
	5	2,485	8.9	102	81	15.8	48	25	5	68	44	6.28	9.2	4.9	0.65	25
	6	2,340	8.9	85	71	15.6	55	22	6	71	38	6.40	9.2	4.9	0.65	25
	7	2,515	7.3	97	72	16.0	52	21	5	68	39	6.40	9.2	4.9	0.65	25
	8	2,570	7.0	99	75	17.6	56	23	5	74	39	6.38	9.2	4.9	0.65	25
Depletion + DCA (20 mg. per day)																
	9	2,380	14	40	48	18.0	80	28	3	105	37	6.10	9.5	2.8	0.54	29
	10	2,015	15	26	35	18.0	76	24	2	98	36	6.20	9.5	2.8	0.54	29
	11	2,550	11	64	65	17.9	72	25	4	93	35	6.20	9.5	2.8	0.54	29
	12	2,495	16	68	71	17.6	77	19	6	90	34	6.43	9.5	2.8	0.54	29
	13	2,040	13	47	41	17.6	72	23	5	90	30	6.17	9.5	2.8	0.54	29
High Na^+ intake (*ca.* 348 mEq. per day)																
J. M.$_2$ Initial													Stool‖			
	1	1,965	60	257	214	10.7	15	4	39	−20	46	7.17	9.2	4.9	0.84	22
	2	2,045	94	284	288	13.3	26	10	25	11	39	6.80	9.2	4.9	0.84	22
	3	3,040	144	383	381	16.1	23	5	57	−29	55	7.17	9.2	4.9	0.84	22
	4	3,355	112	388	390	16.9	28	9	39	− 2	43	6.98	9.2	4.9	0.84	22
	5	2,240	97	280	276	16.6	30	9	30	9	40	6.97	9.2	4.9	0.84	22
Depletion (*ca.* 1 mEq. per day)																
	1	1,770	50	229	183	16.8	35	12	15	32	50	6.82	5.5	3.6	0.46	27
	2	2,665	37	327	230	16.3	30	8	33	5	53	7.03	5.5	3.6	0.46	27
	3	2,230	20	292	221	16.4	31	11	24	18	50	6.90	5.5	3.6	0.46	27
	4	1,960	16	238	177	15.9	33	12	15	30	43	6.80	5.5	3.6	0.46	27
	5	2,330	15	259	219	16.7	40	8	20	28	37	6.97	5.5	3.6	0.46	27
	6	3,285	11	364	281	16.2	34	5	42	− 3	46	7.16	5.5	3.6	0.46	27
	7	2,650	12	305	248	17.1	42	8	24	26	43	7.00	5.5	3.6	0.46	27
Depletion + DCA (20 mg per day)																
	8	1,935	19	192	179	16.3	61	17	6	72	38	6.52	6.4	0.8	0.38	22
	9	2,535	23	271	211	16.7	49	6	33	22	46	7.12	6.4	0.8	0.38	22
	10	2,570	23	299	251	16.3	52	9	31	30	47	7.00	6.4	0.8	0.38	22
	11	1,560	19	205	168	14.5	55	8	17	46	42	6.92	6.4	0.8	0.38	22
	12	1,725	28	176	156	15.5	54	8	20	42	32	6.90	6.4	0.8	0.38	22

* Titratable acid, T.A.

† Total hydrogen, H^+, = (NH_4^+ + T.A. − HCO_3^-).

‡ Phosphate expressed in terms of base requirement for excretion at given pH.

§ Chloride in stool averaged 0.3, 1.1 and 0.6 mEq. per day, respectively, for the three experimental periods.

‖ Chloride in stool averaged 0.9, 0.6 and 0.4 mEq. per day for the three experimental periods, respectively.

¶ Chloride in stool averaged 0.1, 0.4, 0 and 1.0 mEq. per day for the initial and three depletion periods, respectively.

** Chloride in stool averaged 2.0 mEq. per day for the initial period and the first six days of the depletion period and 1.0 mEq. per day for the rest of the experiment.

TABLE II—*Continued*

Subject	Day	Urine											Stool¶			
		Vol.	K^+	Na^+	Cl^-	N	NH_4^+	T.A.*	HCO_3^-	H^+†	PO_4‡	pH	K^+	Na^+	N	Solids
		ml.	*mEq.*	*mEq.*	*mEq.*	*Gm.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*		*mEq.*	*mEq.*	*Gm.*	*%*
R. H.																
Initial																
	1	1,710	96	255	266	14.9	25	12	19	18	29	6.70	7.5	1.5	0.8	36
	2	(specimen lost)											7.5	1.5	0.8	36
	3	2,500	78	336	325	14.9	21	5	31	− 5	32	7.05	7.5	1.5	0.8	36
	4	2,830	88	410	387	16.6	29	5	60	−26	39	7.10	7.5	1.5	0.8	36
	5	2,840	88	368	350	16.0	21	9	37	− 7	35	6.93	7.5	1.5	0.8	36
Depletion (*ca.* 1 mEq. per day)																
	1	2,560	49	245	221	14.7	22	16	13	25	36	6.62	6.7	2.3	0.6	36
	2	1,970	30	261	201	15.5	21	15	14	22	44	6.73	6.7	2.3	0.6	36
	3	2,180	26	235	167	15.0	21	16	18	19	49	6.80	6.7	2.3	0.6	36
	4	2,400	18	288	215	16.5	24	13	21	16	45	6.80	6.7	2.3	0.6	36
	5	2,200	9.4	228	170	14.6	25	14	13	26	35	6.70	5.5	0.8	0.7	30
	6	2,480	12	303	218	15.5	24	8	25	7	49	7.06	5.5	0.8	0.7	30
	7	3,200	13	342	263	15.0	23	5	36	− 8	40	7.17	5.5	0.8	0.7	30
	8	2,440	11	350	285	15.1	24	8	27	5	34	7.02	5.5	0.8	0.7	30
Depletion + DCA (20 mg. per day)																
	9	1,800	17	207	171	14.4	33	17	16	34	33	6.60	6.9	0.7	0.7	28
	10	1,630	24	205	144	14.9	29	17	9	37	40	6.60	6.9	0.7	0.7	28
	11	1,660	25	208	176	15.0	35	12	15	32	35	6.85	6.9	0.7	0.7	28
	12	1,450	24	178	144	14.5	36	13	12	37	22	6.70	6.9	0.7	0.7	28
	13	2,395	33	282	233	15.2	39	5	26	8	46	7.10	6.9	0.7	0.7	28
	14	3,085	36	330	283	15.1	45	6	35	16	34	7.02	6.9	0.7	0.7	28
Depletion																
	15	2,180	10	276	154	13.0	27	1	37	− 9	28	7.35	5.0	4.0	0.7	18
	16	2,820	9.9	381	265	14.0	30	0	55	−25	45	7.40	5.0	4.0	0.7	18
Repletion (215 mEq. K^+ per day)																
	1	2,750	28	591	470	16.4	24	0	85	−61	35	7.48				
	2	3,600	79	666	615	15.3	24	0	72	−48	24	7.40				
J. S.													Stool**			
Initial																
	1	1,250	78	214	218	16.6	40	13	4	49	36	6.00	30	17	2.4	12
	2	2,335	85	292	333	18.1	52	25	5	72	38	6.18	30	17	2.4	12
	3	2,810	88	342	370	16.9	46	17	14	49	42	6.58	30	17	2.4	12
	4	2,260	82	306	325	16.2	47	22	8	61	38	6.36	30	17	2.4	12
	5	1,940	82	316	304	16.7	50	23	7	67	37	6.32	30	17	2.4	12
Depletion (*ca.* 1 mEq. per day)																
	1	2,420	44	232	219	16.8	51	27	5	72	43	6.22	21	26	1.9	15
	2	2,500	27	288	228	16.8	51	23	10	64	50	6.50	21	26	1.9	15
	3	2,275	17	248	203	14.7	50	18	11	57	46	6.62	21	26	1.9	15
	4	2,365	18	296	255	15.7	52	16	13	55	43	6.58	21	21	2.3	16
	5	2,290	12	277	224	15.4	53	17	12	58	48	6.62	21	21	2.3	16
	6	2,930	13	411	348	16.1	53	8	26	35	36	6.90	21	21	2.3	16
	7	2,565	11	294	247	14.1	55	11	20	47	46	6.85	16	18	2.0	15
	8	2,425	15	249	192	19.4	74	22	14	82	64	6.62	16	18	2.0	15
	9	2,055	10	257	198	16.6	54	12	19	48	57	6.88	16	18	2.0	15
	10	2,220	11	296	234	18.4	64	14	15	63	51	6.76	16	18	2.0	15
Depletion + DCA (20 mg. per day)																
	11	2,125	19	242	212	17.8	73	19	8	84	45	6.56	24	4	1.6	17
	12	2,105	22	225	192	18.2	77	17	14	81	51	6.70	24	4	1.6	17
	13	2,060	18	242	220	17.0	80	15	16	79	42	6.68	24	4	1.6	17
	14	2,440	18	275	254	15.9	81	16	20	77	39	6.75	24	4	1.6	17
	15	2,790	17	349	317	15.5	84	8	36	55	40	7.02	24	4	1.6	17
	16	2,090	15	234	212	15.0	80	11	25	66	41	6.82	24	4	1.6	17

corrected for negative nitrogen balance), the smallest deficit being in the subject on normal sodium intake (Table III).

All of the subjects had positive *sodium* and *chloride* balances (Table III). The ratio of sodium retained to chloride retained exceeded the dietary ratio of these two ions in each subject studied. The subject on a normal sodium intake showed a ratio of 2.1. Two of the subjects on high sodium intakes, J. M.$_2$ and R. H., had ratios of 1.7, while the third, J. S., had a ratio of 2.7. Sodium retained exceeded the amount of potassium lost. For the subject on normal sodium intake, the ratio of sodium retained to potassium lost was 2.1. For the subjects on high sodium intake, the ratio was 2.7 in two of the subjects and 1.5 in the third. The greatest positive sodium balance occurred during the DCA period, with drops in stool sodium output as well as in that of the urine.

All of the subjects had negative *nitrogen* balances despite adequate levels of protein intake (Table III). These balances ranged from −3.3 to −38.8 Gm. for the total depletion periods (including DCA) of 12 to 16 days.

Urinary excretion of acid

In three studies (J. M.$_1$, J. M.$_2$ and R. H.) *urinary pH* showed no substantial changes (Table II). In the fourth study (J. S.) urinary pH rose as potassium depletion progressed.

The three major components affecting total acid excretion, namely, ammonium ion, titratable acidity (T.A.) and bicarbonate, showed greater

TABLE III

Cumulative balances of nitrogen and electrolytes *

Subject	Duration	Daily intake, mean and range				Cumulative balance				
		N	K	Na	Cl	N	K	K′	Na	Cl
	days	*Gm.*	*mEq.*	*mEq.*	*mEq.*	*Gm.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*
Normal Na^+ intake										
J. M.$_1$ (69.8–67.7)										
K^+ Depletion										
	8	16.5 (16.4–16.7)	4.8 (0.6–13)	134 (129–140)	94	−10.5	−170	−138	+226	+100
K^+ Depletion + DCA										
	5	16.8 (16.5–17.2)	0.7 (0.7–0.8)	128	94	−11.8	−118	− 83	+371	+190
Total	13					−22.3	−288	−221	+597	+290
High Na^+ intake										
J. M.$_2$ (68.4–68.9)										
K^+ Depletion										
	7	16.8 (16.6–17.1)	0.4 (0.1–0.5)	348 (347–348)	259	− 8.1	−203	−179	+378	+232
K^+ Depletion + DCA										
	5	16.6	0.5	347	259	− 2.8	−149	−141	+578	+315
Total	12					−10.9	−352	−320	+956	+547
R. H. (74.3–72.3)										
K^+ Depletion										
	8	16.4 (16.0–16.9)	1 (0.6–2.5)	348 (348–352)	259	− 2.6	−216	−208	+512	+311
K^+ Depletion + DCA										
	6	16.2 (14.4–16.6)	0.7 (0.5–0.9)	342 (309–349)	254 (230–259)	− 0.7	−201	−199	+625	+362
Total	14					− 3.3	−417	−407	+1137	+673
J. S. (63.7–62.3)										
K^+ Depletion										
	10	16.6 (15.7–17.1)	0.8 (0.5–1.0)	349 (348–350)	259	−26.8	−365	−285	+408	+206
K^+ Depletion + DCA										
	6	17.0 (16.9–17.1)	1 (0.5–1.2)	350 (349–351)	259	−12.0	−253	−217	+497	+129
Total	16					−38.8	−618	−502	+905	+335

* Figures in parentheses after subject initials give body weights in Kg. at the beginning and end of each study.

changes (Figure 2). *Titratable acidity* showed no change or increased slightly after the predepletion period in three of the studies (J. M.$_1$, J. M.$_2$ and R. H.). The fourth study (J. S.) showed some decrease in the excretion of titratable acidity as the depletion progressed. In all of the studies *ammonium ion* excretion increased as depletion progressed and most strikingly so during DCA administration.

The net urinary acid or *total effective hydrogen ion excretion* ($NH_4^+ + T.A. - HCO_3^-$) (18) is shown in Figure 2 compared with the expected excretion as calculated from the predepletion period excretion and the changes in cation and anion intake (the only changes were in potassium chloride and bicarbonate). In general, the net acid excretion was slightly to greatly below the expected value during the potassium depletion prior to DCA administration. During the DCA period the net acid excretion rose in three of the subjects (J. M.$_1$, J. M.$_2$ and R. H.) above the expected value resulting in the retention of bicarbonate. In one subject, J. S., the urine continued to contain less acid than predicted but the decrement was not so great during the period of DCA administration. In R. H., during repletion, more bicarbonate was excreted and acid was conserved.

Symptoms

The subjects felt well, in general, throughout the studies. Some felt tired during the last few days, but they thought that this was due to boredom generated by the routine and confinement required by the study. One of the subjects, J. M., felt headache and nasal congestion

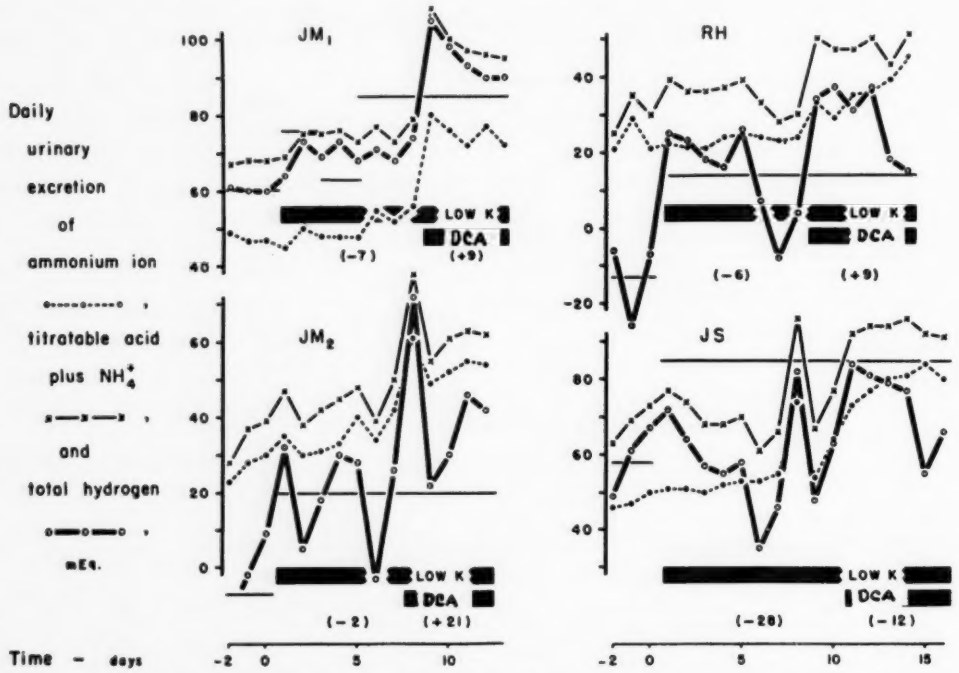

FIG. 2. THE DAILY TOTAL URINARY EXCRETION OF ACID AND COMPONENTS, AMMONIUM ION AND TITRATABLE ACIDITY, DURING POTASSIUM DEPRIVATION AND ADMINISTRATION OF DESOXYCORTICOSTERONE

The total acid excretion, ΔUV_{H^+}, is calculated as $\Delta UV_{NH_4^+} + \Delta UV_{T.A.} - \Delta UV_{HCO_3^-}$. The horizontal lines indicate the total acid excretion predicted from the values of the last three days of the control period and the change in intake of anions relative to cations (the only variation in this level, that in J. M.$_1$, was due to diet change). The values in parentheses in each experimental period indicate the average daily difference between the actual acid excretion and that predicted from the dietary anion-cation balance and the control rate; during K deprivation alone less acid was excreted and DCA reversed or diminished this trend.

in the last three days of the DCA period. Subject J. S. had a sensation of chilliness and muscle ache, without fever, during the sixth and seventh days of depletion; these symptoms were attributed to a mild incidental viral infection.

RESULTS: DERIVED

Relation of potassium deficit to blood CO_2 content

Although only one subject (J. S.) developed an arterial total CO_2 content significantly higher than the upper limit of normal (24 mMoles per L.), the other subjects did show a tendency to develop rising total CO_2 levels in both venous serum and arterialized cutaneous whole blood as the severity of the potassium depletion increased and especially when DCA was given. This tendency is emphasized when CO_2 values are plotted as increments above the last value of the pre-depletion period against the negative balance of potassium (Figure 3).

Analysis of internal transfers of water and electrolytes

Much of the subsequent interpretation depends in large part on the use of the chloride space calculation (17) for an estimate of extracellular fluid volume. The validity of this use has been argued for at least a decade and the authors cannot support either end of the argument from the data of this study. A recent thorough analysis of the chloride space problem by Cheek, West and Golden (19) has provided strong support for the close approximation of extracellular fluid volume in the rat by this method under normal circumstances. How good this approximation is for following sequential changes in human pathological situations is not clear. Possible sequestration of chloride-containing fluid in an atonic bowel may be an objection to the use of the chloride space in the presence of potassium depletion (20). Several roentgen studies of gastro-

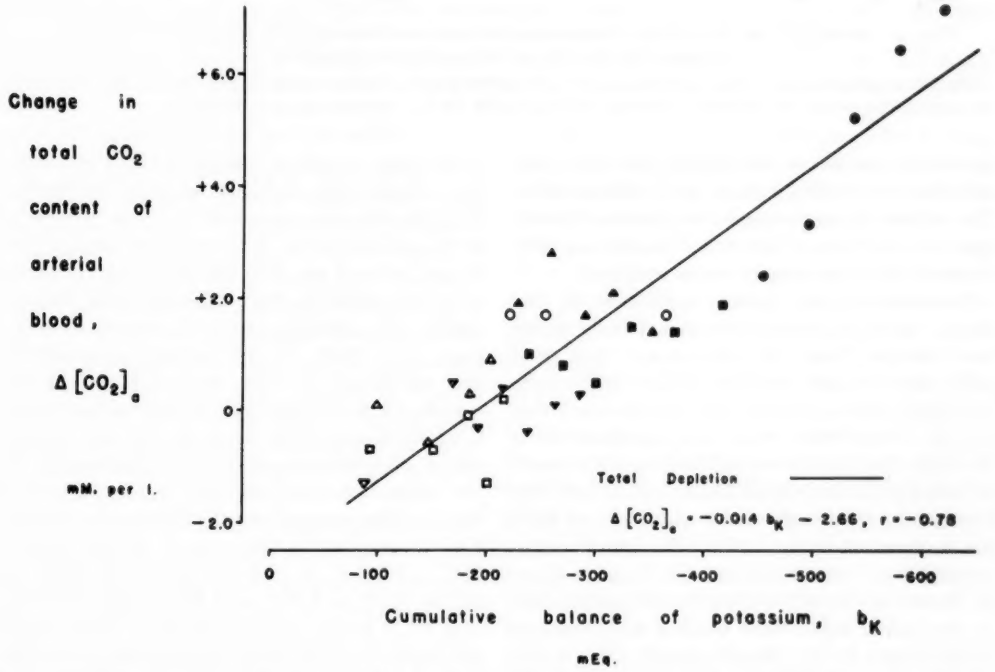

FIG. 3. THE REGRESSION OF CHANGE IN ARTERIAL TOTAL CO_2 CONTENT ON THE CUMULATIVE POTASSIUM DEFICIT

Symbols are for the different subjects, open symbols representing pre-DCA periods and solid symbols representing those of DCA administration.

The negative correlation is good although the changes in arterial CO_2 content are not large.

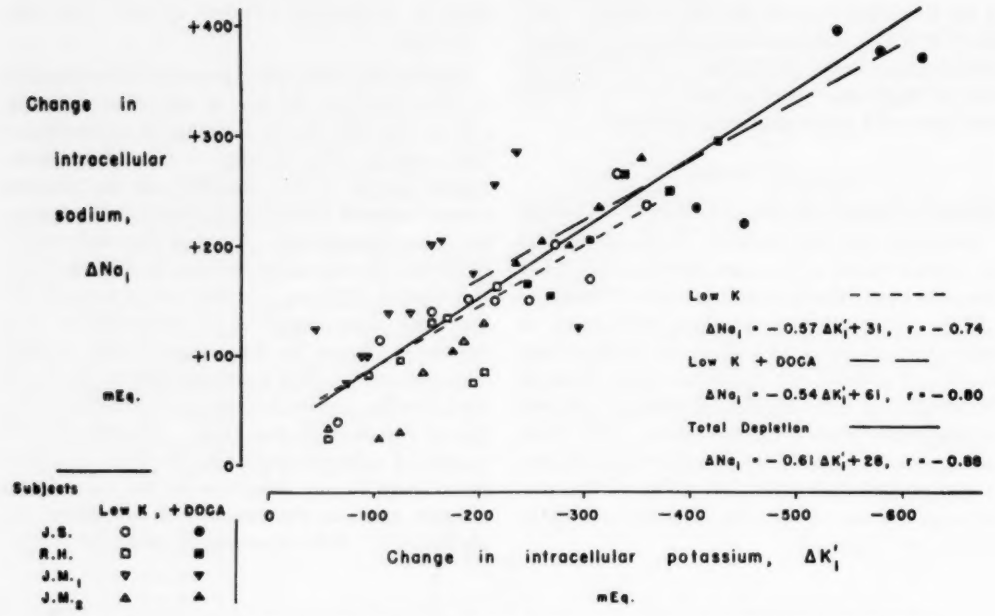

FIG. 4. REGRESSION OF CALCULATED CUMULATIVE INCREASE OF INTRACELLULAR SODIUM ON CALCULATED CUMULATIVE DEFICIT OF INTRACELLULAR POTASSIUM

The relationship is close to that predicted (+2 : −3) by the Darrow findings in rat skeletal muscle (see text) and is the same during potassium deprivation without DCA and with DCA. Symbols are as in Figure 3.

intestinal function in the subjects of a previous experiment (1) failed to show such a disturbance. The authors have proceeded to use the chloride space as an arbitrary theoretical foundation fully aware of the other uncertainties involved.

In assessing the water metabolism in this study, approximations of the total body water were derived from the assumption that total body water weight equalled 74 per cent of the lean body mass at the beginning of the depletion period. Lean body mass was calculated from the daily creatinine excretion during the control periods by the formula of Miller and Blyth (21). Changes in total body water were derived from the formula of Peters (17). The initial extracellular fluid volume was arbitrarily assumed to be 28 per cent of the total body water and changes in the extracellular fluid volume were equated with changes in the chloride space. The difference between total body water and extracellular water (chloride space) was taken as intracellular water. All of the subjects maintained total body water close to normal, one of them (J. S.) showing a slight and probably insignificant decline. The chloride space expanded by 30 to 50 per cent of the original value (an expansion equal to 5 to 10 per cent of the original lean body mass) in all of the subjects, the expansion being greatest during the administration of desoxycorticosterone. As a result, the intracellular water volume was calculated to have decreased by approximately 12 to 15 per cent of its original value. Calculated insensible water losses were similar among the subjects, all of whom had practically the same estimated lean body mass (about 57.0 Kg.). The average losses and their ranges were: 1.45 L. per day in the control period (1.37 to 1.53); 1.28 in the initial potassium depletion period (1.22 to 1.37); and 1.26 in the depletion and DCA period (1.11 to 1.34). These values are higher than for basal subjects because of the normal activity allowed the subjects. The differences among the periods are not statistically significant owing to the small number of data.

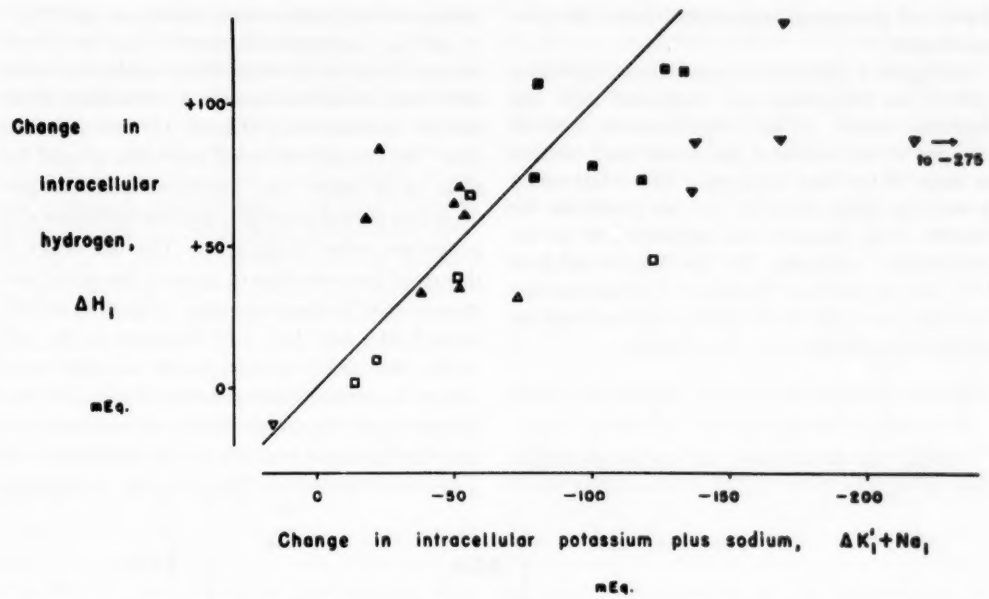

FIG. 5. COMPARISON OF CALCULATED CUMULATIVE INCREMENT OF INTRACELLULAR HYDROGEN WITH THE CALCULATED CUMULATIVE DEFICIT OF INTRACELLULAR POTASSIUM NOT REPLACED BY SODIUM

The line indicates a theoretical 1 : 1 ratio. In three of the subjects such a relationship approximately obtained; in the fourth, J. S., ΔH_i greatly exceeded $-\Delta K_i + \Delta Na_i$ and the data are not plotted (see text). Symbols are as in Figure 3.

Internal transfers of potassium and sodium

In each subject the intracellular phase progressively lost potassium in excess of nitrogen and gained sodium. The quantitative relationships of these exchanges are shown in Figure 4. The regression line with a regression coefficient of 0.61 demonstrates that these subjects as a group showed the phenomenon of replacement of approximately two-thirds of the potassium loss by sodium, as found by Darrow, Schwartz, Iannucci and Coville (2) in rats. This 2:3 ratio was the same during potassium depletion alone and during depletion plus DCA administration.

Internal transfers of hydrogen and bicarbonate

These transfers were derived by the method of calculation previously reported by Elkinton, Singer, Barker and Clark (22); the details of this approach and its validation will not be discussed here. Briefly, movements of hydrogen[2] in one direction with respect to the intracellular phase (ΔH_i) (or equivalent movements of bicarbonate in the opposite direction) are calculated from the change in total amount of extracellular plus red cell bicarbonate ($\Delta HCO_3^-{}_{er}$) corrected for the change in hydrogen in the protein nonbicarbonate buffers of the blood ($\Delta H^+{}_{buf}$) and for the external balance of hydrogen (b_{H^+}):

$$\Delta H_i^+ = \Delta HCO_3^-{}_{er} + \Delta H^+{}_{buf} + b_{H^+}$$

The external balance of hydrogen is derived by comparing the change in urinary excretion of hydrogen (ΔUV_{H^+}), calculated as the sum of changes in excretion of titratable acid plus ammonium ion minus bicarbonate ($\Delta UV_{TA} + \Delta UV_{NH_4^+} - \Delta UV_{HCO_3^-}$), with the change in the acid content of the diet which accompanied the with-

[2] "Hydrogen" as used here refers to both the ionized form and to the vastly larger quantities present in the weak acids of buffer systems; the gaseous form is not implied. Possible changes in CO_2 production do not have significance in this calculation; the rigid control of dietary intake practically excludes changes in acid production (see Reference 22).

drawal of potassium supplement from the prepared diet.

In Figure 5, changes in intracellular hydrogen (ΔH_i^+), so calculated, are compared with the algebraic sums of the simultaneous derived changes in intracellular potassium and sodium in three of the four subjects. The relationship is not far from that of 1:1 as predicted by Darrow and suggests the development of an intracellular acidosis. In the fourth subject, J. S., the increments of cellular hydrogen so far exceeded the loss of potassium against sodium gained that the data are not plotted.

Relations of renal acid excretion, intracellular acid-base changes and extracellular alkalosis

During the development of potassium depletion before the DCA period, extracellular bicarbonate or total buffer anion ($\Delta BB_{er} = \Delta HCO_3^-{}_{er} + \Delta H^+{}_{buf}$) increased (Figure 6), but the simultaneous increase in extracellular (chloride) space led to only small increases in extracellular bicarbonate concentration (Figure 1). At the same time, the cumulative renal excretion of acid ran close to or below that predicted from the predepletion period excretion and the withdrawal of potassium salts (Figure 2). The net effect of this renal response was to increase the calculated intracellular hydrogen content (Figure 6) in Subjects J. M.$_1$ and J. S. In Subjects J. M.$_2$ and R. H., the effect was to make no substantial change in extracellular or intracellular acid-base balance and the extracellular accumulation of total buffer anion was practically attributable to a new equilibrium established in the distribution

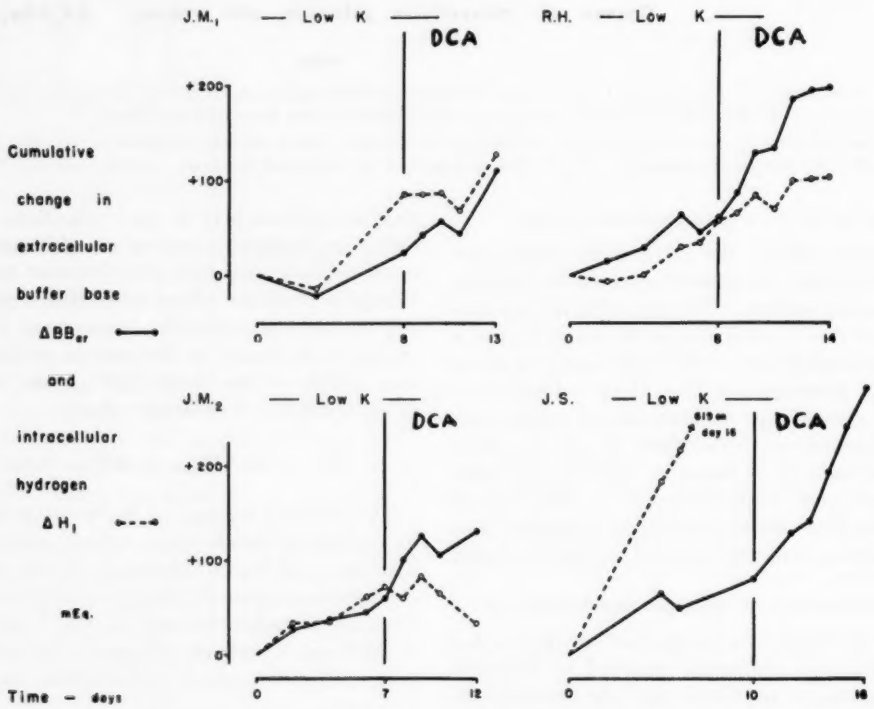

FIG. 6. RELATION BETWEEN THE CUMULATIVE CHANGE IN TOTAL EXTRACELLULAR BUFFER BASE OR ANION (BICARBONATE PLUS PROTEINATE, ΔBB_{er}) AND IN INTRACELLULAR HYDROGEN (ΔH_i)

The difference between the two represents the cumulative renal excretion of acid. When ΔH_i equals ΔBB_{er}, the change in ΔBB_{er} can be attributed to a redistribution of hydrogen ion between intracellular and extracellular compartments. When ΔH_i rises toward or exceeds ΔBB_{er}, the kidney has excreted less acid or more alkali than predicted; when ΔH_i falls toward or below ΔBB_{er}, the converse holds. DCA appears to promote this later change, affecting primarily ΔH_i rather than ΔBB_{er} which remained proportional to the cumulative potassium deficit.

of bicarbonate and hydrogen between intracellular and extracellular compartments.

During the administration of DCA, the renal excretion of acid ran either above that predicted or, in the case of J. S., above the previous values (Figure 2). At the same time, the net result of extracellular accumulation of total buffer anion and increase in chloride space was only a modest additional rise in extracellular bicarbonate concentration in three subjects (J. M.$_1$, J. M.$_2$ and R. H.). In these three subjects, the major effect of this increase in acid excretion was to reduce the absolute intracellular hydrogen content or reduce its rate of increase. In the fourth subject, J. S., a sharper increase in serum bicarbonate concentration took place and the increased renal excretion of acid may have contributed to this because the rate of transfer of hydrogen into cells was essentially unchanged.

The examination of the relation between increments in extracellular total CO_2 concentration (and HCO_3^-) and the deficit of potassium (Figure 3) in the light of the preceding paragraphs suggests that the major effect of the kidney was a change in the intracellular acid-base pattern, and that the extracellular bicarbonate content held a relatively fixed relation to the size of the depletion little modified by renal acid-base excretion.

For Subject J. S. the results of these calculations do not agree entirely with the Darrow hypothesis. The cumulative change in extracellular buffer anion (ΔBB_{er}) shows a fair agreement with the deficit of cellular potassium over sodium $[-(\Delta K_i + \Delta Na_i)]$, the respective values at the end of the depletion period without DCA and at the end of the DCA period being +80 and +120, and +281 and +241 mEq. However, because the urine output of acid consistently ran below that predicted (Figure 2), the calculated changes in intracellular hydrogen (ΔH_i) ran far higher than the calculated cellular cation deficit $[-(\Delta K_i + Na_i)]$, the respective figures for the two periods being +350 and +120, and +619 and +241, and far higher than the accumulation of extracellular buffer anion (Figure 6).

DISCUSSION

Many of the findings of this study agree with those of previous experimental human potassium depletions (1, 13, 14, 23, 24). The degree of hypokalemia in relation to the size of the total depletion, *i.e.*, a serum level of 3.0 mEq. per L. related to a depletion of 300 to 400 mEq., agrees well with findings of Black and Milne (13) and of Scribner and Burnell (25). The absence of definite symptoms (even at a depletion of 600 mEq.) has been noted (13, 14, 23). The expansion of extracellular fluid (chloride space) and inferred contraction of intracellular fluid volume have been described in experimental depletions in human subjects (13, 14) and in comparable depletions of the rat in which NaCl rather than $NaHCO_3$ was the chief salt in the intake as in Group 5 of Cheek and West (11). Hence, these aspects of the problem will not be discussed further.

However, our data should help to clarify the relationship between potassium depletion and extracellular metabolic alkalosis in man. The Darrow hypothesis, as referred to in the introduction and later elaborations of it, is the most inclusive explanation to date of this association. This hypothesis holds that the development of an intracellular potassium deficit and the failure of sodium to entirely replace this deficit lead to an equilibrium shift of hydrogen into cells (or outward shift of bicarbonate) with the development of a lower pH within cells and a higher pH and bicarbonate level in extracellular fluid. The kidney is said to be unable to correct the extracellular alkalosis because the decline in availability of tubular potassium ion for exchange for sodium reabsorption or because an increased hydrogen content of the tubular cells leads the kidney to continue to excrete more acid than required by the extracellular acid-base situation. Recent experimental evidence supports this interpretation. Roberts, Randall, Sanders and Hood (26) found that the administration of potassium chloride to dogs with high pCO_2 levels reduced the elevated tubular reabsorption of bicarbonate without change in the pCO_2. Anderson and Mudge (27) by direct observations *in vitro* of kidney slices from potassium-depleted rabbits found a direct correlation between the potassium content and the bicarbonate content of the slices. These studies were interpreted to show that renal tubular reabsorption of bicarbonate and excretion of hydrogen are augmented by potassium depletion presumably by an increase in acidity of the renal tubular cell.

With respect to the renal maintenance of the abnormal steady state of extracellular alkalosis, our data do not support the above hypothesis, since all our subjects in this study excreted less rather than more hydrogen during potassium depletion alone. This is discussed below. With respect to the internal shifts of cations, however, our derived data, considering the number and nature of the assumptions required, agree reasonably well with the Darrow hypothesis in three of the four studies (J. M.$_1$, J. M.$_2$ and R. H.), *i.e.*, that the intracellular deficit of potassium was replaced by increments of sodium and hydrogen from extracellular fluid in the ratio of 2:1. In the other study, J. S., the calculations would suggest an increment of intracellular hydrogen far in excess of this ratio, though the sodium replacement was approximately two-thirds of the potassium loss.

The type of acid-base analysis used here faces a number of criticisms. There are many possible sources of error in the estimation of change in intracellular hydrogen which have been discussed previously (22), but in the present analysis three primary difficulties are encountered. The first is that of selecting the acid excretion from the predepletion period urinary data which represents the attainment of equilibrium. This point is needed for the estimation of subsequent increments and decrements in acid excretion during depletion. An error of 5.0 mEq. in the selection of this point could lead to an error in the final estimation of cumulative ΔH_i^+ of up to 100 per cent in these studies of duration 12 to 16 days. Later study has shown that the use of the average value for the third, fourth and fifth days of the predepletion period is justified as representing equilibrium (28). Endogenous protein catabolism proceeded at a substantially constant rate throughout each study, in the control and depletion periods; its effect on renal acid excretion, therefore, did not change.

The second primary difficulty faced in this analysis is the validity of the chloride space estimation of extracellular fluid volume. This question has been mentioned and the validity cannot be established further here. It should be noted that the Darrow hypothesis derived from analysis of muscle from potassium-depleted rats is also based on the assumptions implicit in the use of the chloride space. The present analysis is, however, open to the additional errors which may be involved in the estimation of chloride space changes from the cumulative data of the balance technique, and in the uncertainty of use of the chloride space calculation in the total body as contrasted with that in a single tissue. But this analysis lies within the same framework of reference and is pertinent to an investigation of the Darrow hypothesis. Nonetheless, the problem of potassium depletion and metabolic alkalosis still merits a study using a determination of extracellular fluid volume based on some other reference point.

The third question is that of the extent to which entry of sodium into intracellular volumes as estimated here can be considered synonymous with entry of sodium into cellular compartments losing potassium, an assumption applied to this analysis. No further evidence on the soundness of this assumption can be presented from this study and the possibility that some of the retained sodium is associated with bone rather than the intracellular volume of high potassium content cannot be denied.

With regard to the increment in H_i in Subject J. S. which ran far higher than predicted from the Darrow hypothesis, several possibilities can be considered. First, if the acid excretion of the predepletion period had not decreased to equilibrium in the last three days, the estimate of expected acid excretion in the depletion period would be too high. Hence, calculations of retained acid would be erroneously high. This objection has been discussed above. Secondly, if the expected increase in acid excretion in depletion attendant on the decrease in bicarbonate ingestion were met by additional acid excretion via the phosphates of the bowel, this phenomenon could not be detected under the methods used in this study.

Change in extracellular volume, as well as total amount of extracellular bicarbonate or buffer anion, is a factor in determining its final bicarbonate *concentration*. Because the expansion of the chloride space was relatively small in Subject J. S., an increase in total *amount* of extracellular buffer anion only moderately larger than that in Subject J. M.$_2$ and slightly less than that in Subject R. H. (Figure 6) resulted in a

relatively large increase in *concentration* of buffer anion and bicarbonate (Figure 1). However, the failure of renal correction of extracellular alkalosis in the face of continued increments of intracellular acid, in part due to renal retention of acid, above that predicted by the Darrow hypothesis remains unexplained except for the suggestion of a fixed relation of obscure nature between total extracellular bicarbonate and intracellular potassium deficit referred to above.

In spite of the reasonable agreement of the data from Studies J. M.$_1$, J. M.$_2$ and R. H. with the hypothesis which has been discussed, the data from Study J. S. and data appearing in the literature on potassium depletion in animals suggest that this hypothesis may be an excessively simple description of the mode of development of metabolic alkalosis. First, the J. S. study data suggest an intracellular accumulation of hydrogen ion (or decrease in HCO_3^-) far beyond that expected from the potassium deficit not replaced by sodium (*vide supra*) (even allowing for loss of acid via the stool). Of similar import are various data from the literature. In rats treated by Darrow, Schwartz, Iannucci and Coville with DCA and low potassium diets (2), groups of animals with the most severe alkalosis were among those with the smallest gap between potassium lost and sodium retained in muscle. Iacobellis, Muntwyler and Griffin (29) found the development of alkalosis in potassium-depleted dogs when losses of potassium from muscle were exceeded by sodium gain. Gardner, MacLachlan and Berman (7) found no decrease in total muscle fixed cation concentration in potassium-depleted rats; they also noted an increase in inorganic phosphate concentration in the rat muscle. Black and Milne (13) estimated that their human subjects had an intracellular retention of phosphate relative to the potassium lost and found an increase in acid-soluble phosphate in erythrocytes. The possibility that in potassium depletion, amino acids, notably lysine, may function as cation replacement has been presented (30). All of these observations suggest that the increment in intracellular hydrogen ion concentration (7) which apparently develops in potassium depletion parallel to the extracellular metabolic alkalosis cannot be simply described by the discrepancy between potassium lost and sodium gained intracellularly but may also be related to changes in the concentration and valency of intracellular anions and to the functioning of amino acids as cations.

Another aspect of the problem of potassium depletion and metabolic alkalosis which has not been described in an adequate hypothesis is the relation of the acidity-alkalinity of the intake (largely reflected by Na:Cl ratio) to the alkalosis. In the rat, the development of alkalosis in the face of potassium depletion is clearly accelerated by a high ratio of Na to Cl in the diet. Rats depleted of potassium on an intake of NaCl in a 1:1 molar ratio (8) took much longer to develop alkalosis than those on a diet with a ratio of 2:1 (2), and the potassium-depletion alkalosis can be corrected by an increase in the acidity of the diet without potassium replacement or rubidium substitution (12). Muntwyler, Griffin, Samuelsen and Griffith (6) found that the development of alkalosis in dogs was much more closely related to the chloride intake than to the relative loss of intracellular potassium and gain of intracellular sodium. Certainly a distinct species difference in susceptibility to alkalosis between rats and dogs exists (6, 29), perhaps due to a tendency of rats to lose more chloride in stools during potassium depletion (31). Finally, in Subject 1 of Black and Milne (13) the extracellular alkalosis which developed during seven days of potassium depletion was possibly conditioned by the increased intake of fixed cation in relation to fixed anion in the resin-treated milk diet.

The effect of desoxycorticosterone (or like endogenous steroids) on the relative renal excretion of sodium and acid cannot be neglected in considering the pathogenesis of the alkalosis. With the administration of DCA the renal excretion of acid increased sharply above the expected level of excretion (Figure 2), the additional acid excretion being secondary to an increase in sodium reabsorption with an attendant increase in hydrogen ion secretion. Certainly in the presence of evidence of increased adrenal cortical activity very definite metabolic alkalosis may develop with modest potassium losses (32). A striking demonstration of the effect of DCA in precipitating metabolic alkalosis in the face of small potassium deficits and chloride loss was

presented by Moore and associates (16), who suggested the importance of DCA in the blockade of renal correction of alkalosis. However, in the present study the renal effect of DCA appears to have led primarily to an absolute or relative decrease of intracellular hydrogen rather than to a marked increase in extracellular content and concentration of bicarbonate. The increases in extracellular bicarbonate concentration are apparently more closely related and proportional to the additional potassium loss induced by the DCA than to its renal acid-base effect.

The additional conclusions are, first, that an intake of sodium substantially in excess of chloride intake favors the development of an alkalosis, whereas high chloride intakes diminish the likelihood of alkalosis (1). Secondly, the exact relations between increments of intracellular acidity and cation deficits are probably not simply expressed by the difference between potassium lost and sodium gained. The chief physiological problems remaining for intensive study in this area include the reliability of the chloride space estimation as an index of extracellular fluid volume in the circumstances of potassium depletion, the exact role of chloride intake and excretion and a more exact analysis of muscle cation and anion composition.

SUMMARY AND CONCLUSIONS

Four balance studies were done on three normal male human subjects depleted of potassium by dietary deprivation of the ion for periods of 12 to 16 days. The treated milk diet contained less than 1 mEq. of potassium and up to 350 mEq. of sodium per day with a $Cl:HCO_3^-$ ratio of 3:1. During the final five or six days of depletion, desoxycorticosterone was given in doses of 20 mg. per day.

Potassium deficits developed ranging from 288 to 618 mEq. per subject (221 to 502 mEq. when corrected for nitrogen balance), the rate of potassium loss in stool and in urine being accelerated by the DCA. Sodium was retained in excess of chloride. Urinary pH showed only small changes but the excretion of ammonium ion increased; the renal excretion of total acid was diminished relative to the excretion calculated as necessary to maintain external acid balance prior to DCA and was relatively increased during DCA administration. Hypokalemia developed in all four subjects; a slight tendency to extracellular metabolic alkalosis appeared in two subjects and a substantial alkalosis occurred in a third.

Derived data showed about a 2:3 ratio for the exchange of extracellular sodium for intracellular potassium (relative to chloride space) in all subjects and roughly a 1:1 ratio for replacement by hydrogen of the cellular deficit of potassium over sodium in three of the subjects; in the fourth, intracellular retention of hydrogen was greatly in excess of this portion.

It is concluded that potassium depletion due to dietary deprivation of normal human subjects results in: *1*) partial replacement by sodium of the cellular potassium deficit in the approximate ratio of 2:3 as found by Darrow, Schwartz, Iannucci and Coville in rats (2); *2*) a more widely variable shift of hydrogen from the extracellular phase with resultant extracellular deficit of hydrogen or excess of bicarbonate and buffer anions; *3*) a tendency for the changes in renal acid excretion to affect more the acid content of the intracellular rather than of the extracellular phase; and *4*) development of extracellular metabolic alkalosis in terms of a rise in *concentration* of bicarbonate and total buffer anion depending upon the associated change in extracellular volume and the net effect of the above internal ionic shifts.

ACKNOWLEDGMENTS

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STUDIES OF THE DIPYRRYLMETHENE ("FUSCIN") PIGMENTS. I. THE ANABOLIC SIGNIFICANCE OF THE FECAL MESOBILIFUSCIN *

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In the study of bile pigments relatively little attention has been given to the "fuscin" category. Siedel and co-workers (1–3) have contributed much of the information available about these compounds. The term bilifuscin was first used by Städeler (4) to describe a dark brown pigment observed during isolation of bilirubin from cattle gallstones. Siedel has shown that this is a dipyrrylmethene resulting from halving of the (tetrapyrrylmethene) bilirubin molecule, each half retaining one of the vinyl ($CH = CH_2$) groups characterizing bilirubin. According to Siedel and Möller (1), mesobilifuscin, as found in the feces or obtained by oxidation of mesobilirubinogen, is the analogous compound in which the vinyl groups have been reduced to ethyl groups. Theoretically, mesobilifuscin could be formed by schism of bilirubin and subsequent reduction of the resulting bilifuscin, or by primary reduction of bilirubin to its meso-derivatives and subsequent schism. This assumes only a catabolic origin and it is also quite conceivable that dipyrrylmethene pigments of mesobilifuscin type might be elaborated during the anabolic sequences of heme metabolism.

Siedel, Stich and Eisenreich (3) have emphasized that mesobilifuscin occurs normally in the feces partly as a dark brown pigment and partly as a colorless chromogen which is readily converted to the pigment by heat and acidification. Mesobilifuscin does not have a distinct absorption band but exhibits diffuse absorption below 500 mμ. It has not been crystallized. It is readily precipitated as a zinc complex from alcoholic solution. Similar properties characterize the amorphous dark brown pigment accompanying stercobilin in the feces and first designated as copronigrin (5, 6).

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Although the color of normal feces is probably dependent in considerable measure on the presence of the intensely colored "fuscin" pigments, their significance in hemoglobin metabolism has remained a matter of speculation. Since mesobilifuscin can be obtained *in vitro* by oxidation of bilirubinoid compounds, the corresponding fecal pigment has been assumed to be a product of hemoglobin breakdown. Technical difficulties have discouraged attempts to clarify this question. Siedel and Möller (1) described a method of purification of mesobilifuscin from feces, but it is clear that this included varying amounts derived by (artifactual) schism of bilirubinoids during the procedure. This is considered again in the following.

The present study was undertaken to determine the significance of mesobilifuscin in respect to hemoglobin metabolism by means of N^{15} labeled glycine.

MATERIAL AND METHODS

Four individuals were studied using N^{15} glycine according to well established methods (7, 8). The first, C. B., a 55 year old male, was studied eight months after a left radical nephrectomy and splenectomy for hypernephroma. No known hematologic disease existed. The second case, J. P., was a 65 year old male with congenital hemolytic anemia. Mention of this patient was previously included in studies describing the isolation of crystalline N^{15} bilirubin from feces and demonstrating conversion of N^{15} labeled mesobilirubinogen to stercobilin by fecal bacteria (9, 10). Case 3, L. H., a 54 year old female, was a patient with sporotrichosis and reticuloendotheliosis. Despite an eventual fatal outcome the patient's condition was relatively good at the time of the present study; there was no evidence of hemolytic anemia. The fourth subject, S. G., a 29 year old female, was a normal volunteer. Thus three of the four subjects studied had normal or relatively normal hemoglobin metabolism. The life span of the erythrocytes approximated 120 days on the basis of the hemoglobin protoporphyrin N^{15} data.

Glycine was prepared by the method of Schoenheimer and Ratner (11) from potassium phthalimide containing 33 atom per cent excess N^{15} for the first three cases and 62 atom per cent excess for the fourth (normal) subject. All four were given 20 Gm. protein diets for several days before and during the period of glycine administration. The labeled glycine was fed in total quantities of 15 Gm. to the normal subject and 53 Gm. to the three others. It was given over a three day period with equal doses at hourly intervals except for triple doses at midnight and 3 a. m. according to the schedule of Shemin and Rittenberg (7). Blood specimens were drawn at one to four day intervals for the first 16 days and at irregular intervals thereafter. Feces were collected continuously for the first several weeks and were divided into either two or four day periods. After five weeks of study the collections were sporadic in Cases 1 and 3. The exact periods of collection for Cases 1, 2 and 4 are shown in Figures 1, 2 and 3, and for Case 3 in Table I .

TABLE I

Comparison of N^{15} concentration in fecal stercobilin and mesobilifuscin in subjects with normal hemoglobin metabolism, at the time of the early and late stercobilin N^{15} peaks

Case	Day after glycine N^{15}	Atom per cent excess in: Hemoglobin protoporphyrin	Fecal stercobilin or urobilin	Fecal dipyrrylmethene or mesobilifuscin
1	2	0.007	0.382	0.309
3	1–4	0.049	0.651	0.666
3	5–8		0.239	0.182
4	3–4	0.028	0.374	0.693
4	5–6	0.242	0.573	0.391
1	133–134	0.111	0.202	0.024
3	128–129	0.072	0.200	0.057
4	117–120	0.248	0.987	0.126

The hemoglobin protoporphyrin and fecal stercobilin were crystallized according to methods previously described (12, 13). The alcohol—Al_2O_3—water modification (13) was used for the isolation of stercobilin. Case 2 (J. P., Figure 2) had been given Terramycin® for seven days prior to administration of glycine; hence *d*-urobilin and bilirubin rather than stercobilin were isolated, by methods previously described (9, 13). The method used for obtaining mesobilifuscin in the first three cases provided a relatively crude material as compared with the modification of Siedel and Möller's method (1) employed in Subject 4. The dark brown or

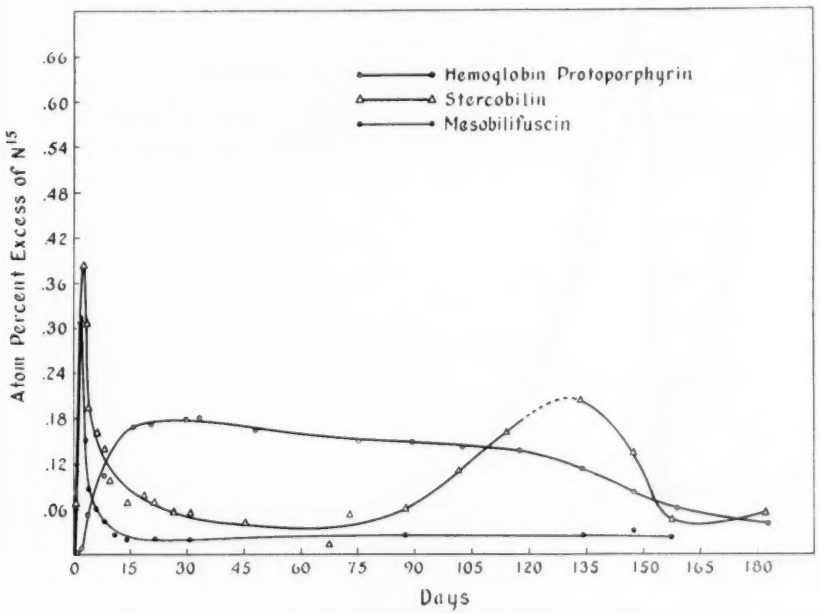

FIG. 1. N^{15} CONCENTRATION IN CIRCULATING HEMOGLOBIN PROTOPORPHYRIN, FECAL STERCOBILIN AND FECAL MESOBILIFUSCIN IN SUBJECT NO. 1 (NORMAL ERYTHROCYTE LIFE SPAN)

Determinations of stercobilin and mesobilifuscin were performed on 24 hour collections of feces during the first 16 days and on 48 hour feces collections thereafter.

almost black precipitate appearing at several stages during the isolation of stercobilin or bilirubin by earlier methods (5, 9, 14) was precipitated by zinc from an alcoholic (zinc acetate) solution in the same manner as previously described for copronigrin. As will be noted in the following, the nitrogen content of the samples thus obtained was considerably lower than the theory for mesobilifuscin or any pure dipyrrylmethene. In all of these the nitrogen determinations were made on the zinc precipitated material; hence the low values are due at least in part to zinc which may have been present in excess. Certain of the more important samples, in point of time, were analyzed for zinc [1] by the method of Valleé and associates (15, 16). These and certain other samples were also recycled according to Siedel and Möller's method after which ordinary and isotopic nitrogen percentages were again determined.[2]

The method used in the fourth or normal subject (S. G.) was a modification of Siedel and Möller's procedure for isolation of mesobilifuscin(ogen) from feces. Most of the fecal samples from Subject 4 were first subjected to the ferrous hydroxide method for stercobilin isolation as previously described (13). With two of the samples (Days 33 to 36 and 41 to 44) the alternative alcohol—Al_2O_3—water method (13) was used. In either event the aqueous filtrate or eluate was extracted repeatedly at pH 4.0 with petroleum ether (30 to 60° B. P.) in order to remove most or all of the urobilinogen group. This was converted in the usual way (13) to the urobilin group and crystallized for N^{15} analysis. The remaining aqueous solution, after petroleum ether extraction, was heated to 80° C. on a boiling water bath and allowed to stand overnight. This results in conversion of colorless chromogen to pigment. The next day it was extracted three times with butyl alcohol containing 2 per cent acetic acid; approximately 75 ml. of butyl alcohol was used for each extraction. The aqueous fraction was then discarded. The butyl alcohol fraction was washed three times with water and filtered through a

[1] The zinc determinations were carried out by Miss Margaret Giebenhain in the Research Laboratory of the Department of Obstetrics and Gynecology, through the courtesy of Dr. J. L. McKelvey.

[2] The ordinary nitrogen determinations were made either in the Microanalytical Laboratory, Division of Organic Chemistry, University of Minnesota, or in the Clark Microanalytical Laboratory, Urbana, Illinois. The atom per cent excess N^{15} was determined in the mass spectrometer through the courtesy of Professor A. O. Nier, Department of Physics, University of Minnesota.

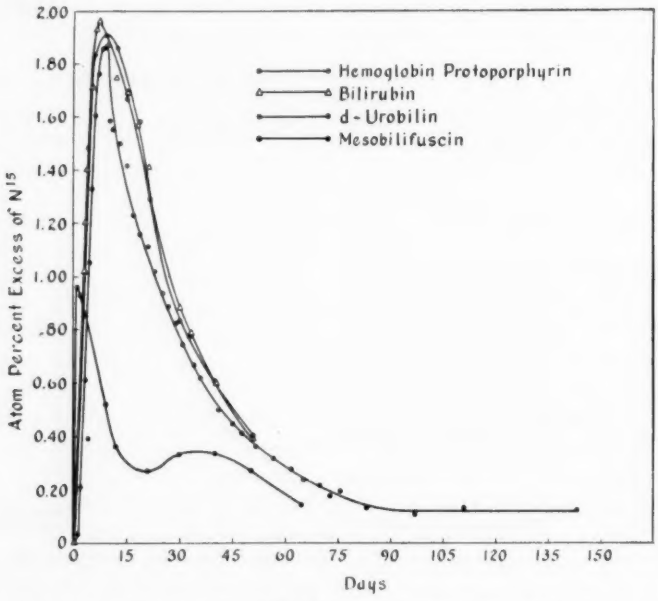

FIG. 2. N^{15} CONCENTRATION IN CIRCULATING HEMOGLOBIN PROTOPORPHYRIN, FECAL BILIRUBIN, FECAL d-UROBILIN AND FECAL MESOBILIFUSCIN IN A PATIENT WITH CONGENITAL HEMOLYTIC ANEMIA (SUBJECT NO. 2)

Determinations were carried out on 24 hour collections of feces during the first 21 days and on 48 hour collections thereafter. The patient had received Terramycin® shortly before the period of study.

butyl alcohol moistened filter paper. It was then concentrated under reduced pressure to less than 5 ml. after which approximately 30 ml. of methyl alcohol and 10 ml. of a saturated alcoholic zinc acetate solution were added. This was allowed to stand overnight or until the zinc complex of the dipyrrylmethene group precipitated. The precipitate was separated by filtration and washed with methyl alcohol. It was esterified with a 20:1 mixture of methyl alcohol: sulfuric acid. Four to 24 hours were allowed for esterification. The total ester was then extracted into chloroform. This was washed once with water, once with 3 per cent ammonium hydroxide and once with 7 per cent sodium chloride. The chloroform was then concentrated to a small volume and was passed through a column of Al_2O_3 first saturated with ethyl ether. The column was first eluted with a 1:10 mixture of chloroform: ethyl ether, then with a 1:3 mixture of chloroform: ethyl ether, then with chloroform alone. Finally the top brown band containing the mesobilifuscin ester was eluted with hot glacial acetic acid. This was mixed with several volumes of chloroform and washed repeatedly with water to remove much of the acetic acid. The chloroform was then concentrated to a small volume and the mesobilifuscin ester precipitated by addition of 10 volumes of petroleum ether. For purposes of comparison with the N data in the first three cases, a fecal sample from Subject 4 (Period 31) was subjected to the method used in obtaining the zinc precipitates as analyzed in Cases 1, 2 and 3. The material was subjected to ordinary N and N^{15} analysis, then esterified and chromatographed according to Siedel and Möller's method, after which the N and N^{15} determinations were repeated.

Rittenberg's procedure (17) was used for collection of nitrogen from the samples.

Since mesobilifuscin has not been crystallized, a comparative study was made of the yields of well defined oxidation products of the fecal pigment and mesobilifuscin prepared by Siedel and Möller's method (1). Strenuous oxidation either with the 20 per cent sulfuric acid-chromic oxide method (18, 19) or the nitric acid method (1) was carried out on four samples of the mesobilifuscin methyl ester isolated from Subject 4 (Periods 10 to 11, 14 through 20, 32 to 33, and 35) and two samples of the zinc complexed mesobilifuscin isolated from Cases 1 and 2. The methyl ethyl maleimide was crystallized by sublimation and the hematinic acid recrystallized from ether petroleum ether. The yields of methyl ethyl maleimide and hematinic acid from each sample were compared with those obtained, after like treatment, of mesobilifuscin methyl ester prepared from mesobilirubinogen (1).

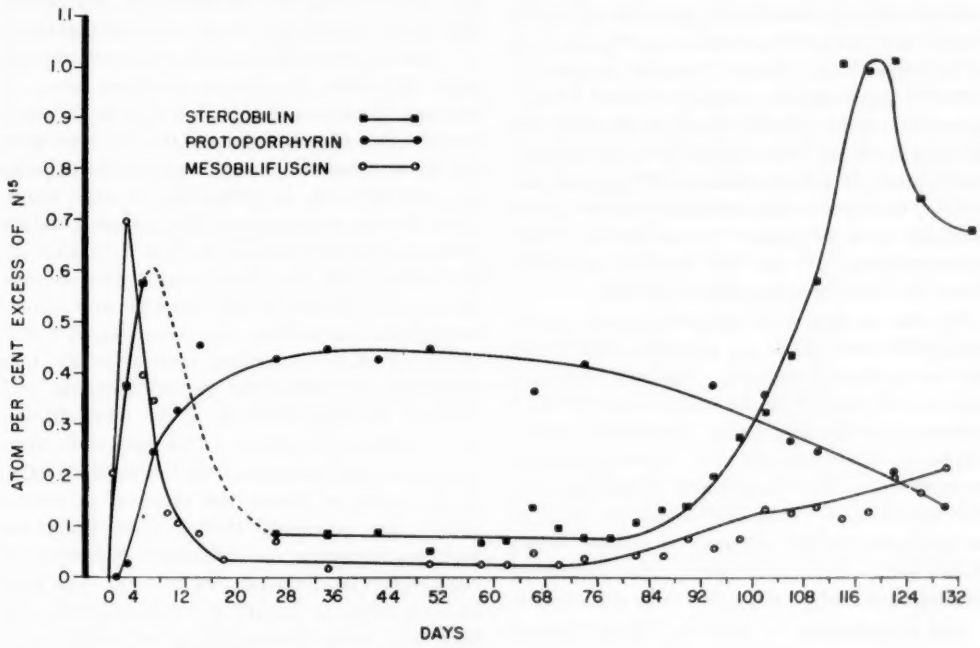

FIG. 3. N^{15} CONCENTRATION IN CIRCULATING HEMOGLOBIN PROTOPORPHYRIN, FECAL STERCOBILIN AND FECAL MESOBILIFUSCIN IN A NORMAL INDIVIDUAL (SUBJECT NO. 4)

Determinations of stercobilin and mesobilifuscin were performed on 48 hour collections of feces during the first 12 days and on 96 hour collections thereafter.

TABLE II

Percentage of N, atom per cent excess N^{15} and Zn before and after recycling

Case	Day after glycine N^{15}	Initial zinc precipitate					Methyl ester obtained after chromatography according to Siedel and Möller (1)		
		N %	Atom % excess N^{15}	Zn %	Ash %	Zn % in ash	N %	Atom % excess N^{15}	Zn % (ash)
	Theoretical	6.45*		30*			8.8		0
1	2	2.7	0.309	28.3	24.6	48.0	6.43		0
1	5	2.44	0.128				6.78	0.130	0
1	133		0.024					0.020	
1	146		0.030					0.040	
2	1	1.29	0.947	87.1	22.1	79.0	2.22	0.638	0
2	4	2.4	0.646				4.6	0.647	
2	10–13	3.97	0.355	27.2	22.6	73.7	5.99	0.466	0
2	49	2.5	0.271	41.1	16.2	98.4	6.95	0.268	0
3	5–8	1.36	0.182				4.99	0.190	
3	126–129		0.057					0.056	
4	8–9						8.96	0.347	
4	107–110	3.42	0.105				8.81	0.133	
4	115–118						8.94	0.126	

* Calculated as the di-zinc complex of mesobilifuscin.

RESULTS

The curves for N^{15} concentration in the circulating hemoglobin protoporphyrin, fecal dipyrrylmethenes and the bilirubinoid pigments for Cases 1 and 2 and Subject 4 are shown in Figures 1, 2 and 3, respectively. Table I permits comparison of the N^{15} percentages in Subjects 1, 3 and 4 (having normal erythrocyte life spans) at the early and late peaks of the stercobilin N^{15}; the former shortly after the administration of N^{15} glycine, the latter at the time of destruction of mature circulating red cells as denoted by the decline of the protoporphyrin N^{15} and the marked secondary rise of N^{15} in fecal stercobilin or urobilin.

The data in these three individuals with normal hemoglobin metabolism are generally similar and may be considered together. The red blood cell survival, determined on the basis of the N^{15} concentration in the circulating hemoglobin protoporphyrin, approximated the normal survival curve described by Shemin and Rittenberg (7). The stercobilin N^{15} data and curves are those to be anticipated in the presence of normal hemoglobin metabolism. The early peak, occurring between four and 12 days, has been described in a normal individual by London, West, Shemin, and Rittenberg (8). It may be derived either from anabolic sources or from destruction of young erythrocytes in the bone marrow or spleen. The second stercobilin N^{15} increase beginning at about 90 days with the peak at 120 to 130 days corresponds with the period of destruction of mature circulating erythrocytes. It is evident that the N^{15} data for mesobilifuscin and stercobilin differ markedly. Only one mesobilifuscin N^{15} peak is observed occurring between two and eight days after the glycine administration. In the normal subject, Figure 3, this peak definitely precedes that of stercobilin and the N^{15} concentration in the mesobilifuscin is already declining while the concentration in stercobilin is still rising. There is some suggestion of this in Case 3 and the precedence is well defined in Case 2 (Figure 2), the patient with hemolytic anemia (see below). By way of contrast, in the subjects with normal hemoglobin metabolism, the stercobilin N^{15} concentration shows a striking increase at the time of red cell destruction but no mesobilifuscin peak is seen. A slight increase in the mesobilifuscin N^{15} is noted in Figure 3. This may well represent artifactual formation from bilirubinoids either in the colon or during the chemical procedure, though the possibility that a small proportion owes its formation to bilirubinoid schism in the body cannot be excluded (see below). The small increase noted in Figure 3 occurs gradually and later than in the corresponding stercobilin curve. Furthermore, it is apparent that the greater part of the mesobilifuscin rise occurs while the N^{15} concentration of stercobilin is decreasing. There was no significant increase in mesobilifuscin N^{15}

at the time of the second stercobilin peak in Cases 1 and 3 (Table I and Figure 1).

It is also noteworthy that in the subjects with normal red cell life span (Nos. 1, 3 and 4) the N^{15} in mesobilifuscin more nearly approaches the base line during the heme (protoporphyrin) N^{15} plateau than does the N^{15} of the stercobilin. If the persistent small elevation of the latter is construed as due to a minor, random destruction of hemoglobin during this period, the lower level of N^{15} in the dipyrrylmethene would be in accord with an anabolic origin.

In Case 2, that of congenital hemolytic anemia (Figure 2), a marked shortening of the red cell survival time is demonstrated by the N^{15} curve for hemoglobin protoporphyrin. In the presence of active hemolysis only one peak is noted in the N^{15} curve for bilirubin and for *d*-urobilin. The mesobilifuscin N^{15} curve reveals an early peak clearly preceding that of bilirubin and *d*-urobilin. The mesobilifuscin N^{15} at first falls more rapidly but levels off between 15 and 30 days, then exhibits a definite secondary rise at the same time that the curves for hemoglobin protoporphyrin, fecal bilirubin and *d*-urobilin are steeply falling.

In the foregoing description of method it was pointed out that the procedure used in the first three cases yielded a crude product of lower total nitrogen percentage than that obtained in the fourth and most detailed study (S. G.). The ordinary nitrogen percentages, with the atom per cent excess N^{15} on the same samples, are given in Table II. The low ordinary nitrogen percentages in the material from the first three cases obviously restrict any conclusions based on these data. The theoretical concentration for a mono-zinc complex is 7.6 per cent N and 18 per cent Zn, for a di-zinc complex 6.45 per cent N and 30 per cent Zn and for the (zinc free) methyl ester 8.8 per cent N. The results of the nitrogen and zinc determinations are given in Table II. The zinc content approximated that of a di-zinc complex in two samples, while in the others zinc was present in excess of the theoretical for such a complex. It is apparent that many of the precipitates analyzed contained excessive zinc due to coprecipitation or as complexes of impurities. The nitrogen analyses both before and after chromatographic purification by Siedel and Möller's method (1) are shown in Table II. It is seen that in the majority of samples analyzed there was essentially no change in atom per cent excess N^{15} before and after application of Siedel and Möller's method. In Case 2 the atom per cent excess N^{15} decreased moderately in one sample and increased slightly in another. The nitrogen content increased consistently after this additional purification recycling, although the theoretical nitrogen percentage was reached in but one sample (No. 4). These observations indicate that most of the nitrogen in the crude samples was in the dipyrrylmethene molecule, the low nitrogen percentage being mainly due to the presence of non-nitrogenous impurities. It is not clear why the nitrogen percentage failed to reach the theoretical after purification of these samples. In the only instance where this did occur (No. 4)

TABLE III

Oxidation of mesobilifuscin (Mbf) from feces as compared with that from mesobilirubinogen (Mbg)

	Yields (mg.) and melting point			
	Methyl ethyl maleimide	M. P.	Hematinic acid	M. P.
1. $H_2SO_4 - Cr_2O_3$ method (18, 19)				
a. 25 mg. from Mbg	5.4	62–65	2.4	110–113
b. 50 mg. from Period 35; Subject No. 4	10.0	63–65	4.0	113–114
c. 50 mg. from Zn complex; Mbf Subject No. 1	3.0	60–65	1.2	98–106
d. 51 mg. from Periods 32, 33; Subject No. 4	7.6	62–65	3.9	112–115
e. 87 mg. from Periods 10, 11; Subject No. 4	12.0	62–65	3.8	113–114
2. Nitric acid method (1)				
a. Siedel and Möller's data (1) 200 mg. Mbf methyl ester from Mbg	14.0	67		
b. 200 mg. Mbf Periods 14–20; Subject No. 4	20.0	63–67	2.1	100–108
c. 200 mg. Zn Mbf; Subject No. 2	6.0	63–68	0.5	110–112

the material was freshly prepared and the purification by Siedel and Möller's method was carried out at once, while the earlier samples had been kept for long periods in the crude zinc precipitate; thus the possibility of a slow complex formation with a non-nitrogenous material must be entertained.

The amounts of methyl ethyl maleimide and hematinic acid obtained after oxidation of mesobilifuscin by two different methods are given in Table III. Use of the sulfuric acid-chromic oxide method resulted in better yields of methyl ethyl maleimide (38 per cent of theoretical) and hematinic acid (22 per cent of theoretical) from the standard (mesobilifuscin prepared from mesobilirubinogen) than did the nitric acid method. The yields obtained from the mesobilifuscin methyl ester isolated from Subject 4 compare favorably with those from the standard by either method, indicating at least a high degree of similarity. In the case of the crude pigments, prepared as the zinc complexes in Cases 1 and 2, the yields were considerably lower than from either the standard or the more highly purified material from Subject 4. Nevertheless the isolation and identification of these oxidation products from the crude pigments, considered in relation to their N percentage and other characteristics, provides strong evidence that their nitrogen was mainly present in a dipyrrylmethene compound. The experiment in the normal subject, No. 4, is regarded as the most decisive, particularly because of the regular use of Siedel and Möller's method with each sample, shortly after the period of collection. The data in Experiments 1 through 3 are less decisive because of the initial method of preparation, nevertheless the results are in general agreement with those of Experiment 4.

DISCUSSION

It has been widely assumed that the naturally occurring fecal dipyrrylmethenes or mesobilifuscins are products of hemoglobin catabolism and are formed by oxidative schism of the bilirubinoids. Siedel, Stich and Eisenreich (3) believed that quantitative measurement of fecal mesobilifuscin might reconcile known discrepancies between the rate of hemoglobin breakdown and fecal urobilinogen excretion. A considerable amount of data collected in various types of anemia reveals that discrepancies of this type are not accounted for by proportionate increases of fecal mesobilifuscin or dipyrrylmethene. These observations are to be described in an ensuing paper of this series. Also, if mesobilifuscin were mainly catabolic, like the urobilinogen group, one would anticipate that after feeding N^{15} glycine there would be a second mesobilifuscin N^{15} peak corresponding to the second stercobilin peak at the time of destruction of mature circulating erythrocytes. It is evident from examination of the present N^{15} curves for mesobilifuscin that a second peak does not occur. Similarly, in the presence of active hemolysis the mesobilifuscin N^{15} falls much earlier than that in the hemoglobin protoporphyrin and bilirubinoid compounds. A secondary rise as the latter continues to fall may be due to reutilization of N^{15} made available from the globin of destroyed hemoglobin. This would accord with the concept of a main anabolic origin of the fecal dipyrrylmethenes, also with that of the amino acid pool (20). Although mesobilifuscin is readily prepared from bilirubinoid compounds and may be formed from them in small amount under appropriate conditions, the present results indicate that the relation of the naturally occurring dipyrrylmethene compounds to hemoglobin catabolism, is insignificant. In this connection, however, the special significance of Bingold's "pentdyopent" (21) must be recognized. This appears to represent a small moiety of dipyrrylmethene formed by the kidney, by schism of heme or bilirubinoids. There is no evidence that it occurs in the feces.

The mesobilifuscin N^{15} curves exhibit an early peak, as seen in Figures 1, 2 and 3. As already noted, this clearly precedes the early stercobilin peak in Subjects 2 and 4 (Figures 2 and 3) and this is suggested in Case 3 (Table I), though here the data are not decisive. In Figures 2 and 3 it is seen that the N^{15} concentration in mesobilifuscin falls earlier and more rapidly than in the corresponding stercobilin N^{15} curve. The early concentration of N^{15} in mesobilifuscin together with the lack of any marked N^{15} concentration at the time of mature erythrocyte destruction indicates that the dipyrrylmethene pigments are mainly anabolic in origin. This concept is also supported by certain clinical observations, to be published separately, which indicate that mesobilifuscin excretion is better correlated with erythropoietic ac-

tivity in the bone marrow than with hemoglobin destruction.

The slight gradual rise in the mesobilifuscin N^{15} curve at 100 to 130 days in the normal subject (Figure 3) merits consideration. The curve is a continuous gradual upward slope with the greatest increase actually occurring when the N^{15} concentration in stercobilin is declining. It is unfortunate that the N^{15} determinations were discontinued at 130 days when the mesobilifuscin N^{15} value had not yet commenced to fall. This was due to delay of many weeks in the isolation and N^{15} analyses. Nevertheless, it is clear that the stercobilin N^{15} peaked at about 120 days and had fallen considerably when the experiment was broken off. Thus, even had there been further increase of mesobilifuscin N^{15} it could not well have been ascribed to hemoglobin destruction. The small sustained increase associated with and following the stercobilin N^{15} peak may be explained on the basis of reutilization of N^{15} made available from the globin of destroyed hemoglobin, as discussed in the foregoing. It is also possible that some part of this minor increase is due to (artifactual) conversion of bilirubinoid to dipyrrylmethene. It should be emphasized that an earlier peak in the mesobilifuscin curve was not overlooked, for each consecutive four day collection of feces was analyzed throughout this period.

It should be re-emphasized that the fecal pigment(s) isolated in the first three cases was relatively crude with a nitrogen percentage considerably lower than the theoretical concentration for a dipyrrylmethene compound. Thus, conclusions based on these data alone would be hazardous. However, when Siedel and Möller's procedure (1) was used from the outset, as in Case 4, the total nitrogen content agreed regularly with the theoretical and the yields from oxidative degradation compared favorably with those from standard preparations. Since the configuration of the mesobilifuscin curve is similar in all four experiments, the validity of the data in the first three is considered to be enhanced, especially in view of the fact, already discussed, that strenuous oxidation provided methyl ethyl maleimide and hematinic acid in amounts indicating that the nitrogen was mainly contained in dipyrrylmethenes.

The means by which mesobilifuscin might be derived from anabolic sources have not been determined. In the broader sense one must consider derivation from a pigment pool or "complex" such as suggested by Whipple (22) in 1922. This would require that a certain amount of bilirubinoid or other pyrrol pigment or precursor be manufactured in excess of that used in heme synthesis and that the excess be converted to mesobilifuscin or similar dipyrrylmethenes. A more specific concept in this regard involves the theory of a tripyrrylmethene intermediate on the pathway from porphobilinogen to porphyrin. The tripyrrylmethene theory was first suggested by Turner (23), more recently by Bogorad and Granick (24) and Shemin, Russell and Abramsky (25). The latter investigators, in particular, have emphasized this possible pathway, pointing out that differing dipyrrylmethanes could thus be provided to account for the formation of the porphyrin isomers. By the same token, an overproduction and excretion of excess dipyrrylmethanes, on this basis, is easily envisioned. This would also be true in respect to the dipyrrylmethane intermediaries postulated for the biosynthesis of uroporphyrin III in the differing concepts of Treibs and Ott (26) and of Bullock, Johnson, Markham and Shaw (27). The dipyrrylmethenes which might be formed, either by oxidation of excessive dipyrrylmethanes or by schism of a hypothetical tripyrrlmethane, might be expected to have acetic and propionic B substituents rather than methyl, ethyl and propionic as found in pure mesobilifuscin, according to Siedel, von Pölnitz and Eisenreich (2). It is quite conceivable, however, that acetic and propionic groups are decarboxylated in part to methyl and ethyl groups, in which event the differences in elementary analysis might well be undetected, especially as the fecal mesobilifuscin has not thus far been crystallized. Siedel and coworkers (2, 3) believe that it is excreted in the form of polymers of varying chain length which may account for the variations in solubility of different fractions and the failure to crystallize.

SUMMARY AND CONCLUSIONS

1. The relation of fecal dipyrrylmethene compounds or mesobilifuscin to hemoglobin metabolism has been investigated in four human subjects with the aid of N^{15} labeled glycine. A modification of Siedel and Möller's method (1) of

partial purification of mesobilifuscin was employed. This included preliminary removal of urobilinogen to prevent addition by oxidative schism to the preformed dipyrrylmethene moiety.

2. The lack of significant concentration of N^{15} in mesobilifuscin at the time of destruction of mature circulating erythrocytes when the stercobilin N^{15} was greatly elevated is presented as evidence that mesobilifuscin is mainly anabolic in origin rather than a product of hemoglobin breakdown.

3. The early incorporation of N^{15} in mesobilifuscin with a peak which often precedes the early stercobilin N^{15} peak also indicates that mesobilifuscin is derived principally from anabolic sequences. The lower N^{15} values for mesobilifuscin during the plateau of the protoporphyrin N^{15} in the circulating erythrocytes are in accord with this concept.

4. The possible mechanisms of mesobilifuscin derivation are considered.

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STUDIES OF THE DIPYRRYLMETHENE ("FUSCIN") PIGMENTS. II. THE CONTRASTING RATIOS AND SIGNIFICANCE OF THE FECAL UROBILINOGEN AND MESOBILIFUSCIN IN CERTAIN ANEMIAS *

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In the preceding communication (1) evidence was described indicating that the fecal dipyrrylmethene or "fuscin" compounds are derived mainly from anabolic sequences on the pathway to heme, rather than oxidative schism of tetrapyrryl bilirubinoids from destroyed hemoglobin. Using N^{15} glycine, it was shown that N^{15} was rapidly incorporated in mesobilifuscin (Mbf) with production of an early peak and that there was little or no significant concentration of N^{15} in Mbf at the time of destruction of mature circulating erythrocytes when the stercobilin N^{15} was greatly elevated. It is recognized, however, that under appropriate conditions Mbf is derived by oxidation of bilirubinoid compounds and that minor fractions of the dipyrrylmethene group in the excreta may originate in this fashion. Under normal conditions the feces contain chromogens and pigments of both anabolic and catabolic origin, *i.e.*, compounds of mesobilifuscin(ogen) type elaborated principally during heme synthesis, and urobilinogen derived, at least mainly, from hemoglobin destruction. It would be anticipated, therefore, that the amount of fecal Mbf as compared with urobilinogen might vary significantly in differing hematologic disorders.

The present study was undertaken to compare the excretion of mesobilifuscin and urobilinogen in the feces of normal subjects and in patients with various anemias.

MATERIALS AND METHODS

A total of 140 individuals were studied. Of these, 19 were normal subjects, either medical or nursing students or hospital personnel. The remainder comprised 32 patients with hemolytic anemia, four with megaloblastic anemia, nine with hyporegenerative anemia, nine with anemia due to rheumatoid disease, 27 with anemia due to various other causes including infection, uremia, iron deficiency, cancer and refractory anemia, 20 with parenchymal liver disease including cirrhosis and hepatitis and 20 with obstructive biliary tract disease.

Collections of feces were made over four or eight day periods in the majority of instances, although in 44, as will be indicated, random or short period samples were analyzed for urobilinogen and mesobilifuscin. Where the four day collection was adequate (at least 350 Gm.) the per diem amount was calculated; otherwise the values are given in mg. per 100 Gm. Urobilinogen was determined by the method of Schwartz, Sborov and Watson (2). After extraction of the urobilinogen from the initial acidified filtrate, the latter was used for determination of Mbf. The method was the modification of Siedel and Möller's procedure (3) described in the preceding paper for isolation of Mbf. Following elution of the methyl ester from the Al_2O_3 column, the hot glacial acetic acid eluate was filtered and diluted. The optical density of this solution was then determined in the Evelyn colorimeter using a 490 filter. The standard was prepared from mesobilirubinogen by oxidation with lead tetraacetate as described by Siedel and Möller (3). In the preceding paper of this series it was shown that the fecal pigment obtained with this method corresponds fully with Siedel and Möller's fecal mesobilifuscin in terms of solubility and chromatographic behavior, spectral characteristics, nitrogen percentage and products of oxidative degradation. For purposes of determining the reproducibility of results with this method, multiple analyses for Mbf were performed by different technicians on fecal samples from six subjects (Table I). The individual variation in urobilinogen and Mbf excretion was examined by analyses of multiple four day samples in 15 subjects, multiple short period samples in three subjects and four day samples followed by one or more short period samples in three subjects (see under Results).

Red blood cell survival was studied in 43 cases using Cr^{51} according to the ascorbic acid modification (4) of the usual method (5–7). Sternal bone marrow aspiration studies were performed in 52 cases.[1] Bone marrow studies were not performed in any of the normal subjects;

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[1] Carried out in the Special Hematology Laboratory at the University of Minnesota Hospital. We acknowledge gratefully the cooperation of Dr. R. Dorothy Sundberg and her associates in the bone marrow studies.

TABLE I

Reliability of method for determination of mesobilifuscin as revealed by repeated analyses of the same samples of feces by different technicians (six samples, two to six analyses of each)

Subject	Analysis No.....	Mesobilifuscin 1	2	3	4	5	6
		mg./100 Gm.					
36		38.3	42.3				
42		38.4	43.2				
52		12.0	11.6	11.6	12.2	11.8	11.7
85		19.8	20.0	19.2	18.6	20.4	18.0
106		12.5	11.4				
119		0.3	0.3				

the normal values had been well established previously (8).

Twenty-four hour urine collections were made in seven of the normal subjects and in 11 patients with parenchymal liver disease. The amount of urobilinogen in these samples was determined by the above-mentioned method (2). Another aliquot of the same urine sample was used for the determination of mesobilifuscin. The urine was first acidified with glacial acetic acid to pH 5 petroleum ether to remove all, or nearly all, of the urobilinogen. The unheated aqueous fraction was then extracted twice with approximately 75 ml. of butyl alcohol. From this point the method was identical with that described above for feces. The presence or absence of bilirubin was determined by the Harrison strip test (9). In two cases the urine bilirubin was quantitated by the method of Malloy and Evelyn (10).

RESULTS

The values obtained for fecal urobilinogen, fecal mesobilifuscin, the urobilinogen/mesobilifuscin (U/Mbf) ratio, the $T_{1/2}$ of Cr^{51} and the myeloid-erythroid volume and percentage of normoblasts

TABLE II

Urobilinogen and mesobilifuscin in the feces, U/Mbf ratio and erythrocyte survival in 19 normal subjects

Subject	Sex	Age	Feces: Urobilinogen	Feces: Mesobilifuscin	Feces: U/Mbf	$T_{\frac{1}{2}}Cr^{51}$
			*mg./day or mg./100 Gm.**	*mg./day or mg./100 Gm.**		*days*
1	M	24	197	17.5	11.3	27.0
2	M	24	150 {175† 125}	13.2 {15.8 10.5}	11.4 {11.0 12.1}	29.5
3	M	22	133 {146 119}	12.2 {14.5 9.9}	10.9 {10.0 11.9}	25.0
4	M	28	111 {124 98}	8.3 {8.8 7.8}	13.4 {9.2 10.0}	26.0
5	M	22	153 {161 145}	12.9 {12.4 13.4}	11.9 {13.0 10.8}	26.5
6	F	27	87	10.5	8.2	26.0
			110*	13.1*	8.5	
			78*	9.8*	8.0	
7	F	23	149 {140 158}	16.1 {16.0 16.2}	9.2 { 8.7 9.7}	28.0
8	F	21	93	7.5	12.4	28.0
9	F	20	70	7.4	9.3	25.0
			104*	12.0*	8.7	
10	F	21	149	11.8	12.6	26.5
11	F	20	83	7.7	10.8	27.0
12	F	28	99 { 74 118}	7.3 { 6.0 8.3}	13.6 {12.3 14.2}	
13	M	29	92	9.0	10.2	
14	M		110*	9.0*	12.2	
15	M	38	92*	8.1*	11.3	
16	M		138*	12.5*	11.0	
17	F		88*	8.5*	10.3	
18	F		72*	6.5*	11.1	
19	M	40	70*	7.2*	9.7	
Normal range			70–197	7.3–17.5	8.2–14.2	25–29.5
Mean and standard deviation			120 ± 37.4	10.9 ± 3.42	11.1 ± 1.44	

* Numbers indicated by an asterisk denote quantitations in mg. per 100 Gm.; numbers without an asterisk denote quantitations in mg. per day.

† The determinations in brackets relate to separate samples either random, as indicated by the asterisks, or four day collections, the average being given at the left in each instance.

in concentrated smears of the sternal bone marrow, in each of 19 normal subjects and in 121 patients with anemia are shown in Tables II through VII. The ranges of the per diem excretion of Mbf (in those cases where feces collections were adequate) and the U/Mbf ratios in 19 normal subjects and 94 patients with anemia (those cases of which there are sufficient numbers to classify) are represented graphically in Figure 1. In the group of normal subjects (Table II) the average daily excretion of urobilinogen was lower than previously reported from this laboratory (11). This may be explained, at least in part, by the inclusion of a greater number of female subjects in whom the red cell mass and, consequently, the urobilinogen excretion are normally less than in the male. The average daily excretion of Mbf and the U/Mbf ratio ranged, respectively, from

TABLE III

Urobilinogen and mesobilifuscin in the feces, U/Mbf ratio, erythrocyte survival and erythropoiesis in 32 patients with hemolytic anemia

Subject	Sex	Age	Feces			Bone marrow		Remarks
			Urobilinogen	Mesobilifuscin	U/Mbf	$T_{\frac{1}{2}}Cr^{51}$	Myeloid erythroid volume	Normoblasts	
			*mg./day or mg./100 Gm.**	*mg./day or mg./100 Gm.**		*days*	%		
20	M		1,306 {960† 1,652}	162 {138 186}	7.9 {7.0 8.8}		15.0	77.4	Idiopathic
21	M	3	519	63	8.2		2.5	71.6	Idiopathic
22	M	57	1,040*	184*	5.7	12	Increased	38.6	Hodgkin's disease
23	F	16	630 {590 671}	223 {140 305}	2.8 {4.2 2.2}		13.5	28.8	Sub. myel. leukemia
24	F	46	238 {276 200}	84 {112 56}	2.8 {2.4 3.6}	12			Idiopathic
25	M	63	1,166	112	10.4		64.5	56.4	Idiopathic
26	F	66	331 {236 426}	38.6 {28.5 48.7}	8.5 {8.3 8.7}		6.0	36.2	Idiopathic
27	M		1,321	150	8.8				Idiopathic
28	M		609	74	8.2				Idiopathic
29	F		251	57.5	4.4				Idiopathic
30	F	25	731	98.9	7.4	15.5	50.0	72.1	Erythro. porphyria
31	F	32	339	40	8.5				Idiopathic
32	M	3	450*	83*	5.4		3.0	62.2	Idiopathic
33	M	8	122	11.9	10.0		7.0	57.0	Idiopathic
34	M	34	320	36	9.0		9.5	78.0	F. hemol. anemia
35	F	6	603* {720* 486*}	70.8* {81.6* 60.0*}	8.5 {8.8 8.1}		12.0	34.0	F. hemol. anemia
36	M	5	410* {334* 486*}	35.6* {29.0* 42.3*}	11.4 {11.5 11.4}				F. hemol. anemia
37	F	6	3,400*	800*	4.5		8.0	42.8	F. hemol. anemia
38	M	11	1,367* {1,860* 874*}	159* {208* 110*}	8.6 {8.9 7.9}				F. hemol. anemia
39	M	4	651	105	6.2		7.0	25.0	F. hemol. anemia
40	M	7	219	28	8.0				F. hemol. anemia
41	F	70	707	146	4.8	12			Cirrhosis
42	M	54	304*	38.4*	7.8				F. hemol. anemia
43	F	5	114	87	1.3		13.0	64.8	Idiopathic
			300*	192*	1.6				
44	M	13	446	53.1	8.5				Sub. lymph. leukemia
45	M	65	1,330*	120*	11.1				F. hemol. anemia
46	F	52	428	49	8.7				Atypical myel. leuk.
47	M	70	378	64	5.9		12.5	55.4	F. hemol. anemia
48	M		518	98	5.3				Idiopathic
49	M	72	252	37	7.0		17.0	65.2	Idiopathic
50	F	16	291	33.8	8.6		4.0	43.5	Idiopathic
51	M	67	190	22	8.6	16.5	5.5	31.8	Hypersplenism
Mean and standard deviation					7.3 ± 2.41				

* Numbers indicated by an asterisk denote quantitations in mg. per 100 Gm.; numbers without an asterisk denote quantitations in mg. per day.

† The values in brackets relate to separate samples, either random, as indicated by asterisks, or four day collections, the average shown at the left.

7.3 to 17.5 mg. per day and from 8.2 to 14.2. Moderate variations in the quantities of urobilinogen and Mbf excreted in consecutive four day or short period samples in eight normal subjects (Table II) and thirteen patients (Tables III through VI) are seen. However, the U/Mbf ratio remained relatively constant in a given individual, varying no more than 2.2 in 20 of 21 subjects with multiple determinations. The one exception (Case 60) will be considered again in the following.

The $T_{1/2}$ of Cr^{51} of 25 to 29.5 days in the normal subjects (Table II) agrees with previous reports using the same method (12). The myeloid-erythroid volume in the bone marrow ranges from 5 to 8 per cent normally, and the normal percentage of normoblasts is considered to be approximately 20 (8).

A sharp contrast to the findings in normal subjects is seen in patients with hemolytic anemia (Table III and Figure 1). In most of these cases the fecal urobilinogen was elevated; however, in eight of 32 patients the urobilinogen was less than 300 mg. per day. In the latter cases the hemoglobin levels were considerably decreased; when urobilinogen values were calculated in relation to the total circulating hemoglobin as "apparent wastage" (13, 14), they were found to be significantly elevated. It should be noted that three of these latter cases (Nos. 33, 40 and 43) were anemic children, aged five to eight, in whom the total circulating hemoglobin values were quite

TABLE IV

Urobilinogen and mesobilifuscin in the feces, U/Mbf ratio, erythrocyte survival and erythropoiesis in anemia

Subject	Sex	Age	Feces			$T_{\frac{1}{2}}Cr^{51}$	Bone marrow		Remarks
			Urobilinogen	Mesobilifuscin	U/Mbf		Myeloid erythroid volume	Normoblasts	
			*mg./day or mg./100 Gm.**	*mg./day or mg./100 Gm.**		*days*	%	%	
					Megaloblastic anemia				
52	M	62	161	18.6	8.6	20	6.0	37.2†	Post-gastrectomy
53	M	82	390*	49.0*	8.0		4.0	36.2†	Pernicious anemia
54	M	65	1,082*	204.0*	5.3		13.0	38.0†	Pernicious anemia
55	F	72	201	98.0	2.0	21	3.0	26.4†	Pernicious anemia
					Hyporegenerative anemia				
56	F	16	13	0.66	20.0		1.5	1.0	
57	M	6	17	0.53	32.1		2.0	25.8	
58	F	67	108	5.8	18.6				
59	F	42	109 {7.8‡	2.5 {2.9	43.6 {2.7		0.5	5.6	Panmyelophthisis
60	F	35	17.2 {18	3.03 {2.2	5.7 {8.2	29			
			30	{6.5	{4.6				
61	F	17	40	3.0	13.3		0.5	47.4	Cong. hypopl. anemia
62	F	50	190	10.8	17.0	14	Trace	33.0	Atyp. myel. leukemia
63	F	50	135	16.8	8.0	19	1.0	7.6	Aleu. myel. leukemia
64	M	69	25*	2.5*	10.0		2.0	2.4	Atyp. myel. leukemia
					Anemia secondary to rheumatoid disease				
65	F	71	139 {194	16.8 {22.8	8.3 {8.5	25			
			{85	{10.8	{7.9				
66	F	47	50	8.5	6.0	24	1.5	40.6	
67	M	54	90	5.3	17.0	25	6.0	10.7	
68	F	52	30	2.6	11.5		15.0	32.4	
69	F	47	34	1.1	30.0		12.0	13.3	
70	F	56	19	0.66	29.0	26.5	17.0	50.6	
71	M	43	40	0.94	42.0	25			
72	F	23	160	22.7	7.0	27	35.0	41.4	
			138*	17.4*	7.0				
73	M	50	245*	32.0*	7.7		6.0	21.8	

* Numbers indicated by an asterisk denote quantitations in mg. per 100 Gm.; numbers without an asterisk denote quantitations in mg. per day.

† Erythrocyte precursors.

‡ The values in brackets relate to determinations on individual four day collections for which the average is given at the left.

small. The values for mesobilifuscin were generally elevated; in but one case (No. 33) was the Mbf within the normal range. Furthermore, the increase of Mbf was often relatively greater than the increase in urobilinogen. This was reflected by the U/Mbf ratio which in 15 cases fell below the normal range. Statistical analysis of these data to determine the probability that 15 of 32 cases would have a U/Mbf ratio less than 8.0 reveals that $p < 0.001$. The $T_{1/2}$ of Cr^{51} was markedly shortened in five cases in which survival studies were performed. Normoblastic hyperplasia was apparent in all 18 cases in which bone marrow examinations were made. When therapy was effective in abolishing a hemolytic anemia, the values for Mbf fell to levels within or below the normal range and the U/Mbf ratio rose to normal or above normal. Data from three cases (Nos. 22, 30 and 43) illustrating this change are given in Table VIII.

A similar pattern of pigment excretion with a tendency toward increased urobilinogen, increased Mbf and normal or decreased U/Mbf ratio is seen in megaloblastic anemia (Table IV, Figure 1). It has previously been shown that a large proportion of the fecal urobilinogen in this disease is derived from sources other than destruction of mature circulating red cells, perhaps by diversion of heme pigment or immediate precursors to production of bile pigment in the presence of ineffective heme synthesis (13, 15). The fecal Mbf was increased out of proportion to the increased urobilinogen. The U/Mbf ratio was markedly lowered in two and at the lower limit of normal in the other two cases. Red cell survival was slightly shortened, with the $T_{1/2}$ Cr^{51} being 20 and 21 days in

TABLE V

Urobilinogen and mesobilifuscin in the feces, U/Mbf ratio, erythrocyte survival and erythropoiesis in 27 patients with anemia due to other causes

Subject	Sex	Age	Feces			Bone marrow		Remarks
			Urobilinogen	Mesobilifuscin	U/Mbf	$T_{\frac{1}{2}}Cr^{51}$	Myeloid erythroid volume	Normoblasts	
			*mg./day or mg./100 Gm.**	*mg./day or mg./100 Gm.**		*days*	%	%	
74	M	68	143	1.3	110.0	25	Increased	5.4	Chronic myel. leuk.
75	F	64	70	4.2	16.6	19.5	4.5	10.6	Macroglobulinemia
76	M		185* [103†	1.6* [0	115.0				Cutanea tarda porphy.
77	M		388* [40	10.8* [0	36.0				Interm. acute porphyria
78	F	70	111 [190	0.0 [0	∞	24	19.0	54.7	Refractory anemia
79	F	64	127 [124	33.0 [0	3.8	29	9.5	54.5	Iron deficiency
80	M	54	124* [96	25.0* [0	5.0	19.5			Uremia
81	M	69	59*	9.0*	6.5		10.0	38.9	Metastatic cancer
82			168*	60.0*	2.8				Cutanea tarda porphy
83	F	25	160*	30.0*	5.4		6.0	29.0	Lead intoxication
84	F	16	80*	14.0*	5.7		6.0	26.8	Pancytopenia, pregnancy
85	M	51	190	21.6	8.8		24.5	0.2	Chronic myel. leukemia
86	F		180*	16.0*	11.2		38.5	9.8	Chronic myel. leukemia
87	M	40	331	41.4	8.0				Chronic myel. leukemia
88	F	48	531*	42.4*	12.5				Subacute myel. leuk.
89	F	19	235*	22.8*	10.3		22.0	20.0	Hodgkin's disease
90	M	27	107	13.0	8.4				Hodgkin's disease
91	F	84	70.5	7.5	9.4	31.5			Multiple myeloma
92	M	64	560	67.5	8.3		1.0	16.9	Macroglobulinemia
93	F	45	208	22.8	9.1	23.0	2.5	19.2	Refractory anemia
94	M	86	110*	13.8*	8.1	31.5			Iron deficiency
95	M	76	92*	13.0*	7.0	29.5	1.5	12.2	Iron deficiency
96	M	50	56	5.4	10.3				Uremia
97	F	46	50 {61 {39	5.7 {6.7 {4.6	8.8 {9.1 {8.4	29.5	8.5	16.8	Uremia
98	M	56	134	10.4	13.0		25.0	6.0	Tuberculosis
99	M	70	322*	23.6*	14.0		2.0	32.8	Macrocytic anemia
100	F	5	604*	68.8*	8.7				Infectious mono.

* Numbers indicated by an asterisk denote quantitations in mg. per 100 Gm.; numbers without an asterisk denote quantitations in mg. per day.

† The values in brackets relate to determinations on a series of four day collections for which the average value is given at the left.

two patients studied; this appears to agree with earlier observations (13, 14) and with the findings of London and West using N^{15} (15).

Values for fecal Mbf in patients with hyporegenerative anemia (Table IV, Figure 1) differed remarkably both from the normal and from patients with hemolytic anemia and megaloblastic anemia. Seven of nine patients studied showed Mbf values which were well below the normal range. While fecal urobilinogen tended to be decreased in this group, it should be noted that four patients had normal urobilinogen values (either as a result of transfusion or hemolysis) and two of these had distinctly low Mbf values. Even in the presence of decreased urobilinogen, the Mbf was disproportionately low. The U/Mbf ratio was elevated in six cases; it was low in one patient (No. 60), the only one in whom significant variation was seen with multiple determinations. In Case 60 the Mbf was uniformly decreased, but the urobilinogen was disproportionately low to a varying degree. Subsequent studies of this remarkable case, to be described separately, have consistently shown an unexplained disappearance of bilirubin. One patient, E.H., a 42 year old woman with drug toxicity, was studied over a 24 day period. She presented the clinical picture of progressive bone marrow failure. Blood transfusions were given intermittently throughout this period. The results of this study are shown in Table IX. Despite fecal urobilinogen values in the normal range, the Mbf decreased with each successive four day period and finally disappeared in the last eight days.

The pigment excretion in patients with anemia secondary to rheumatoid disease (Table IV, Figure 1) was similar to that seen in hyporegenerative anemia. The fecal urobilinogen and Mbf were normal or decreased. The U/Mbf ratio was normal or increased. Red cell survival was normal. Bone marrow studies, however, did not reveal reduced erythropoiesis. In fact, four of seven cases in which marrow studies were performed, manifested normoblastic hyperplasia. The possible significance of this is considered again in the following.

Table V includes data from 27 cases with anemia due to other causes. Fecal collections were

TABLE VI

Urobilinogen and mesobilifuscin in the feces, U/Mbf ratio, erythrocyte survival and erythropoiesis in 20 patients with parenchymal liver disease

Subject	Sex	Age	Feces			$T_{\frac{1}{2}}Cr^{51}$
			Urobilinogen	Mesobilifuscin	U/Mbf	
			*mg./day or mg./100 Gm.**	*mg./day or mg./100 Gm.**		*days*
101	F	29	178*	3.0*	59.0	
102	F	50	20.5	0.17	120.0	
103	M	50	140	7.6	19.0	22.0
104	F	48	47 {49† 45	2.2 {2.16 2.25	21.4 {22.6 20.0	24.0
105	M	47	154*	1.6*	96.0	
106	M	61	22	8.5	2.6	21.0
107	M		148*	10.3*	14.4	
108	M	47	326*	18.5*	17.6	
109	M		42	2.3	18.1	
110	F		66	1.1	60.0	
111	F	48	311	1.0	311.0	12.0
112	M		7.5	0.6	12.5	
113	F	59	155	8.4	18.4	
114	F	47	138	6.4	21.5	
115	F	20	32	1.2	26.6	
116	M	42	14.4	9.0	1.6	
117	M	68	86.5 {50 123	4.8 {2.8 6.9	18.0 {17.8 17.8	
118	M	57	60	3.1	19.6	22.5
119	F	60	178*	0.3*	593.0	17.0
120	M	24	308*	6.1*	51.0	

* Numbers indicated by an asterisk denote quantitations in mg. per 100 Gm.; numbers without an asterisk denote quantitations in mg. per day.

† The values in brackets relate to individual four day collections for which the average is given at the left.

adequate for per diem measurements in only 13 of this group. Nevertheless, the U/Mbf ratio could be determined in all. The ratio was high in five patients (Nos. 74 through 78): of these two had normoblastic hypoplasia of the bone marrow; two had hepatic porphyria (one cutanea tarda and one intermittent acute); and one was a patient with refractory anemia in whom the bone marrow appeared hyperplastic. The urobilinogen values were normal in four of these five cases and borderline in one (388 mg. per 100 Gm. feces). The U/Mbf ratio was low in six cases (Nos. 79 through 84). In all six the urobilinogen values were within the nomal range, and in the four cases in which bone marrow studies were done, normoblastic hyperplasia was present. The remaining 16 cases had normal U/Mbf ratios. Of the nine in which per diem measurements were possible, the fecal Mbf was normal in three, slightly decreased in two and slightly elevated in two. Bone marrow studies in four of the latter cases revealed normal or slightly reduced erythropoiesis. In two cases both Mbf and urobilinogen were markedly elevated (Nos. 87 and 92). The bone marrow was studied in one (No. 92) and appeared hypo-

TABLE VII

Urobilinogen and mesobilifuscin in the feces and U/Mbf ratio in 20 patients with biliary obstruction

Subject	Sex	Age	Feces: Urobilinogen	Feces: Mesobilifuscin	U/Mbf
			*mg./day or mg./100 Gm.**	*mg./day or mg./100 Gm.**	
121	F		0.24	0.0	∞
122	F	81	65.0*	7.6*	8.5
123	F	54	0.84	0.0	∞
124	M	72	6.8	0.0	∞
125	M		0.16	0.0	∞
126	F		9.5	0.0	∞
127	F		6.0*	0.0*	∞
128	M	59	3.7	0.21	17.6
129	F		0.8	0.0	∞
130	M		6.5	0.0	∞
131	F		8.6	0.0	∞
132	F	24	52.0	1.3	40.5
133	M	75	1.3	0.0	∞
134	M	75	115.0	12.8	9.0
135	F	67	1.02	0.0	∞
136	F		13.0	0.4	33.0
137	F	4 mos.	1.1*	0.0*	∞
138	F	4 mos.	0.5*	0.0*	∞
139	M	75	3.9	0.0	∞
140	M	65	2.7*	0.0*	∞

* Numbers indicated by an asterisk denote quantitations in mg. per 100 Gm.; numbers without an asterisk denote quantitations in mg. per day.

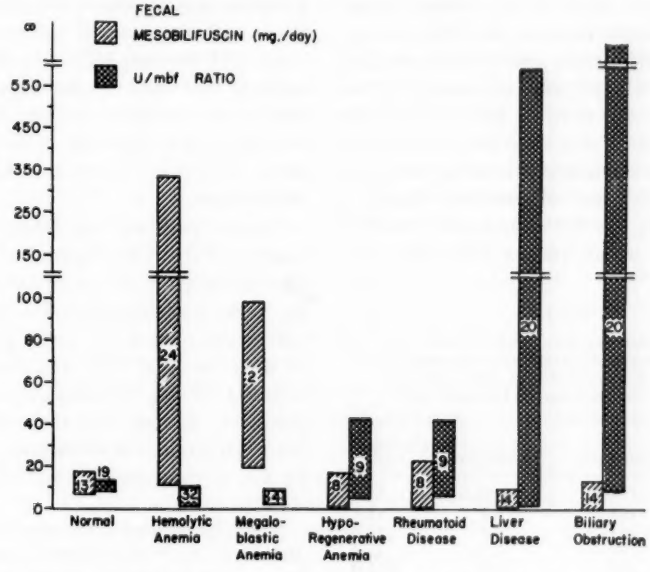

FIG. 1. FECAL MESOBILIFUSCIN AND U/MBF RATIO IN NORMAL SUBJECTS AND IN PATIENTS WITH ANEMIA

Numbers within the bars indicate the number of cases in each group.

TABLE VIII

Change in fecal mesobilifuscin and U/Mbf ratio with treatment of hemolytic anemia

Subject	Sex	Age	Time	Urobilinogen	Mesobilifuscin	U/Mbf
				*mg./day or mg./100 Gm.**	*mg./day or mg./100 Gm.**	
22	M	57	Presplenectomy	1,040*	184.0*	5.7
			Postsplenectomy	247	13.6	17.5
			1 Year postsplenectomy	124	13.9	9.3
30	F	25	Presplenectomy	731	98.9	7.4
			Postsplenectomy	30	3.4	9.0
43	F	5	Pretreatment	114	87.0	1.3
			Cortisone therapy	43	4.0	11.0

* See footnote to Table VII.

plastic; this will be considered again in the following.

In the presence of parenchymal liver disease (cirrhosis or hepatitis) the values for fecal Mbf were usually low, while the urobilinogen showed considerable variation. In the 20 patients in this series (Table VI, Figure 1) the values for fecal urobilinogen were within the normal range in eight, slightly elevated in three and decreased in nine. However, fifteen cases showed definitely reduced values for Mbf and three of the remainder were at the lower limit of the normal range. This disproportionate decrease of Mbf was reflected in the U/Mbf ratio which was quite high in 17 cases. The U/Mbf ratio was normal in one case and definitely low in two. In the latter cases (Nos. 106 and 116) Mbf excretion was normal but urobilinogen was disproportionately decreased. Of interest are the red cell survival studies in six cases. The $T_{1/2}$ of Cr^{51} was in the hemolytic range (12 days) in one patient (No. 84); urobilinogen was slightly elevated in this case (311 mg. per day), but Mbf was markedly decreased (1.0 mg. per day). In the other five cases the $T_{1/2}$ of Cr^{51} was moderately shortened (17 to 24 days), but the urobilinogen was not elevated. These findings are in accord with the earlier study of Jones, Weinstein, Ettinger and Capps (16). In marked contrast to the decreased fecal excretion of Mbf was the finding of increased amounts of Mbf [2] in the urine of 10 out of 11 patients as compared with seven normal subjects (Table X). Bilirubin was present in the urine of the three patients with the greatest values for mesobilifuscin (Nos. 111 through 113). In these cases it is not unlikely that some of the pigment was bilifuscin derived by oxidation of bilirubin. No bilirubin was detected in the urine of the other seven cases, three of which, however, exhibited excesses of urobilinogen.

TABLE IX

*Panmyelophthisis following para-aminosalicylic acid and isoniazid; independence of fecal urobilinogen and mesobilifuscin and disappearance of Mbf with progressive bone marrow failure in Patient E. H.**

Time	Urobilinogen	Mesobilifuscin	U/Mbf ratio
	mg./day	*mg./day*	
10/20–23	103	5.7	18.1
10/24–27	83	5.7	14.6
10/28–31	64	2.5	25.6
11/1–4	145	0.9	161.0
11/5–8	91	0.0	∞
11/9–12	170	0.0	∞

* The patient was a 42 year old woman.

Twenty patients with biliary obstruction were studied (Table VII, Figure 1). The fecal U/Mbf ratio was high in all but two of this group. In 11 the fecal urobilinogen value indicated complete biliary obstruction (< 5 mg. per day) and in 10 of these no fecal Mbf was demonstrable; (Case 101 had 3.7 mg. urobilinogen and 0.21 mg. Mbf per day). In nine cases there was incomplete biliary obstruction (urobilinogen > 5 mg. per day). In five of these no Mbf was demonstrated in the

[2] Although obtained by the same adaptation of Siedel and Möller's (3) method as applied to feces, the material thus far analyzed is obviously impure and may be complexed with a non-nitrogenous urinary constituent, as the percentage of nitrogen (4.0 to 5.0 per cent) is less than the theoretical for Mbf ester, *i.e.*, 8.8.

feces, while in the other four small quantities were present.

DISCUSSION

Evidence described in the preceding paper (1), obtained by comparison of isotope labeled fecal stercobilin (or urobilin) and mesobilifuscin, after feeding N^{15} glycine, indicated that the latter is derived mainly from anabolic sources. The present data are in accord with that concept.

If the fecal Mbf were derived mainly from schism of bilirubinoid compounds as formerly postulated, one might anticipate that in different disease states the fecal urobilinogen and Mbf would vary in the same direction and to about the same degree. In other words, the U/Mbf ratio would be expected to remain essentially the same. Actually, as shown in the present study, the U/Mbf ratio varies greatly in different types of disease.

On the other hand, if the dipyrrylmethene pigments such as mesobilifuscin are derived mainly from anabolic sources, then their amount might often reflect the magnitude of heme synthesis. Thus, in hemolytic anemia, where red cell production is greatly enhanced, Mbf is also increased to a marked degree, often indeed to a relatively greater extent than the urobilinogen, so that the U/Mbf ratio is low. Similarly in pernicious anemia, with its megaloblastic arrest in the bone marrow and ineffective heme synthesis (see above), the fecal Mbf is high and the U/Mbf ratio low. This is not surprising in view of the observations mentioned previously, that in this disease a large proportion of the fecal urobilinogen is also derived from sources other than mature circulating red blood cells. In the hyporegenerative or "refractory" anemia, as a rule, very little fecal Mbf is found while the urobilinogen is often normal or increased, the latter being due either to a hemolytic factor, to blood transfusions, or both. Thus, the U/Mbf ratio is often high. A striking example of this is seen in Table IX. Observations in certain other cases of anemia (Table V) also support this concept. In most of these cases the rates of erythrocyte production and destruction are in balance. Thus, the U/Mbf ratio is normal. Bone marrow studies in seven of the 11 cases with abnormal U/Mbf ratios show increased erythropoietic activity in all four cases with low U/Mbf ratios and decreased erythropoietic activity in two cases with high U/Mbf ratios. Several additional cases of anemia, not included in the Tables, have been studied recently. These have also shown a general parallelism between heme synthesis, erythropoiesis and Mbf excretion in the feces. Thus, the present data indicate that the magnitude of effective or attempted heme synthesis is more significant than hemoglobin destruction in determining the amount of fecal Mbf.

TABLE X

Excretion of urobilinogen, mesobilifuscin and bilirubin in the urine of normal subjects and patients with parenchymal liver disease

Subject	Urobilinogen	Mesobilifuscin	Bilirubin
	mg./day	*mg./day*	*mg./day*
	Normal subjects		
4	1.1	1.2	0
6	1.4	2.8	0
10	1.6	3.0	0
12	0.6	2.9	0
13	0.8	1.8	0
14	1.8	1.0	0
15	0.9	1.8	0
	Parenchymal liver disease		
107	4.9	18.9	0
108	6.0	24.0	0
109	0.75	6.5	0
110	0.2	10.1	0
111	69.0	60.1	4+
112	5.4	46.3	63.0
113	30.9	40.0	16.2
114	0.2	16.2	0
115	0.8	12.9	0
116	1.2	8.1	0
117	12.0	1.4	Not determined

Exceptions to this rule are seen, however. In some cases Mbf is reduced and the U/Mbf ratio is high, despite apparently normal or increased erythropoietic activity. Apart from the cases of liver and biliary tract disease, these findings have been encountered in patients with rheumatoid disease (Table IV, Figure 1), and in one patient (Case 78) with refractory anemia who had normoblastic hyperplasia of the bone marrow but no demonstrable Mbf in either feces or urine. In this situation one may consider the possibility that the immediate precursors of the dipyrrylmethene or "fuscin" compounds are being more efficiently utilized. It is conceivable that in association with an increased erythropoiesis, hemoglobin synthesis might proceed at a relatively slower rate but more efficiently

so that no excess of precursors is available for Mbf formation. This would imply a more complete utilization in porphyrin synthesis, with little available for the excess dipyrrylmethene of the feces. The possibility might also be considered that some fraction of the fecal dipyrrylmethene, at least in some cases, is derived along the biosynthetic pathway to a heme other than that of the hemoglobin formed in the normoblast. It is true that in the two cases of hepatic porphyria (Nos. 76 and 77) the fecal Mbf was rather low, despite the strong likelihood that the metabolic disturbance in these cases is related to the biosynthesis of an hepatic heme or hemes, rather than hemoglobin. It is interesting to speculate that in this situation the excessive porphyrin and/or precursor formation might deplete the total "pigment pool" of the body to such an extent that Mbf elaboration related to hemoglobin synthesis might be significantly reduced. Nevertheless it is quite possible that under other abnormal circumstances there might be excessive dipyrrylmethene production unrelated to hemoglobin synthesis. In the case of lead poisoning (Case 83) with abnormal porphyrin synthesis, excessive amounts of fecal Mbf and a low U/Mbf ratio were found.

In some cases conflicting results are found in the same disease state. Cases 96 and 97 with uremia show decreased fecal urobilinogen and Mbf and normal U/Mbf ratios, while Case 80, also uremia, shows increased fecal Mbf and a low U/Mbf ratio. The latter patient had a moderately shortened red cell life span ($T_{1/2}$ of Cr^{51} was 19.5 days) but a bone marrow biopsy was not done and unfortunately the per diem urobilinogen excretion was not determined. It is quite possible that a hemolytic element was important in this case.

In two cases of macroglobulinemia the findings are also at variance with each other. In one (No. 75) Mbf excretion was decreased and the U/Mbf ratio was high, while in the other (No. 92) both urobilinogen and Mbf were definitely increased and the U/Mbf ratio was normal. The bone marrow appeared hypoplastic in both. The basis of this difference is not clear.

Patients with liver disease usually excrete small amounts of fecal Mbf and exhibit high U/Mbf ratios. The ratio may be low if the fecal urobilinogen is disproportionately reduced as in Cases 106 and 116 (see above). A likely explanation of the reduced fecal Mbf is that it cannot be excreted normally by a damaged liver and is diverted to the urine. This concept is supported by the consistent increases in urinary "mesobilifuscin" noted in Table X. At the same time, comparison of the data in Tables II, VI and X reveals that the total Mbf in urine and feces in the cases of hepatic disease is often considerably greater than that encountered normally. To what extent this increase is due to oxidative schism of bilirubin or urobilinogen, possibly in the kidney or urine, or to anabolic excesses, has not been determined.

SUMMARY AND CONCLUSIONS

1. The amounts of mesobilifuscin and urobilinogen in the feces have been compared in a group of normal subjects and in cases of various types of anemia and liver disease. The results in many of the cases were further compared with erythrocyte survival and erythropoiesis, with the aid of Cr^{51} and bone marrow studies.

2. The range of values for mesobilifuscin in normal human feces is 7 to 18 mg. per day and that for the urobilinogen/mesobilifuscin (U/Mbf) ratio is 8 to 14.

3. Conditions associated with increased erythropoietic activity, such as hemolytic and megaloblastic anemias, were generally associated with increased fecal mesobilifuscin (Mbf) and urobilinogen (U) and normal or low U/Mbf ratios. Conversely, cases of hyporegenerative anemia usually had decreased Mbf and high U/Mbf ratios. This was also true in rheumatoid disease despite increased normoblastic activity in the bone marrow.

4. The observations summarized in Conclusion 3 accord better with the concept that the native fecal Mbf or its colorless chromogen is mainly anabolic, formed during the biosynthesis of heme, rather than a product of heme destruction and bilirubinoid schism.

5. In cases of liver disease, the fecal Mbf is disproportionately small and the U/Mbf ratio high, while the urinary (crude) Mbf is consistently elevated. The extent to which the latter represents diversion of Mbf ordinarily excreted

in the bile, or derivation from bilirubin or urobilinogen in the kidneys or urine, has not been determined.

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THE EFFECT OF CHANGES IN VENTILATION AND PULMONARY BLOOD FLOW ON THE DIFFUSING CAPACITY OF THE LUNG*†

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The "pulmonary diffusing capacity" for carbon monoxide (D_{LCO}) is of interest as a measure of the extent and permeability of the alveolar-capillary interface. However, its use for this purpose is subject to three major types of error: *1*) undetected influences of other physiologic factors, such as changes in pulmonary blood flow, the volume of air in the lungs and the distribution of inspired air; *2*) disregarded characteristics of the test substance, such as altered rates of chemical combination of carbon monoxide with hemoglobin at different oxygen tensions; and *3*) artifacts due to the test procedure itself, such as those introduced by deliberate respiratory maneuvers.

These factors assume different proportions in different methods of measurement.

For the single breath technique, some of these factors, such as the volume of air in the lungs (1–3) and the rate of combination of hemoglobin with carbon monoxide, have been extensively studied (4, 5). For the steady state methods, many remain to be assessed.

Before applying a steady state method to a variety of physiological problems, it seemed pertinent to us to delineate some factors which could affect the D_{LCO}.

The present study considers specifically the effect of minute ventilation and pulmonary blood flow on the diffusing capacity for carbon monoxide determined by a steady state method.

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GENERAL PRINCIPLES

The diffusing capacity of the lung for carbon monoxide, the pulmonary blood flow and the minute ventilation all increase during exercise. In order to determine if the change in minute ventilation or the change in pulmonary blood flow or both, influence D_{LCO}, three different approaches were used: *1*) Minute ventilation was increased more than pulmonary blood flow by exercising subjects with only a limited capacity to augment cardiac output; *2*) minute ventilation was again increased in excess of a change in pulmonary blood flow by having subjects undergo voluntary hyperpnea; and *3*) blood flow through each lung was varied simultaneously in such a way that blood flow increased in one and decreased in the other while minute ventilation remained virtually unchanged.

SUBJECTS AND METHODS

Rest and exercise. Thirteen subjects with mitral stenosis on the basis of rheumatic heart disease were studied both at rest and during exercise. For the sake of comparison, D_{LCO} was also measured in 21 normal subjects at rest (Table I) and an additional 15 normal subjects were studied both at rest and during exercise (Table II).

These studies involved the simultaneous measurement of minute ventilation, cardiac output and D_{LCO}. Cardiac output was measured by the Fick principle, using oxygen as the test gas and entailed: *1*) open circuits for the administration of inspired gas as well as for the collection and sampling of expired gas; *2*) cardiac catheterization for sampling of pulmonary arterial blood; and *3*) cannulation of a brachial artery for the sampling of mixed arterial blood. For the estimation of D_{LCO}, 0.1 per cent carbon monoxide in air was substituted for ambient air for four to six minutes of the test periods at rest and during exercise.

Exercise was performed in the supine position using a special pedal-pulley device attached to the fluoroscopic table. In all subjects but four, a single level of exercise was used; in these four, exercise was continued for a second period at a greater intensity. Midway through the gas collection period, samples of systemic and pulmonary

arterial blood were drawn for the measurements of oxygen contents, as well as carbon dioxide and carbon monoxide tensions.

The steady state method for measuring D_{LCO} proposed by Filley, MacIntosh and Wright (6) was modified in two respects: *1)* The carbon monoxide tension of expired gas was measured by an infrared physical analyzer[1] and *2)* the carbon monoxide content of arterial blood was measured by the method of Allen and Root (7); this value was used to calculate the tension of carbon monoxide in arterial blood by means of the Haldane relationship (8). For this calculation, it was assumed that in normal subjects, the mean difference in oxygen tension between alveolar gas and arterial blood is approximately 10 mm. Hg. Since the removal of CO from physical solution may continue after blood has left the pulmonary capillary, it is clear that arterial pCO can only underestimate the mean back pressure of carbon monoxide in the pulmonary capillary (4). In this study, therefore, arterial pCO is used as a minimal estimate of mean capillary pressure for carbon monoxide.

The pCO_2 of arterial blood was determined from the line charts of Van Slyke and Sendroy (9). For this measurement, the blood CO_2 content and oxyhemoglobin saturation were determined by the method of Van Slyke and Neill (10) and pH by the MacInnes-Belcher glass electrode. The oxygen and carbon dioxide contents of expired gas were measured in a micro-Scholander apparatus (11).

To relate pulmonary arterial pressures to D_{LCO}, blood pressures were recorded by means of Statham transducers coupled with a multichannel oscilloscope recording apparatus.[2]

Voluntary hyperpnea. In 10 of the normal subjects and four of the patients with rheumatic heart disease, the observations at rest and during exercise were supplemented by similar measurements during a period of voluntary hyperpnea. The level of ventilation during hyperpnea was set to correspond to that which was reached spontaneously during exercise. In order to avoid depletion of carbon dioxide and to preserve a steady state, 3 or 5 per cent CO_2 was added to the inspired mixture in accord with the level of minute ventilation. The actual sequence was as follows: After measurements at rest and during exercise, the subjects underwent a final period of hyperpnea which consisted of breathing 3 to 5 per cent CO_2 in air for 14 minutes followed by an additional four minutes of the same inspired mixture, plus 0.1 per cent CO. During the last two minutes, blood and expired gas samples were collected in the usual way for the measurement of pulmonary blood flow and D_{LCO}.

Unilateral occlusion of one pulmonary artery. In six subjects with either a normal cardiorespiratory system or

[1] Liston-Becker Division of Beckman Instrument Co., Stamford, Conn.

[2] Electronics for Medicine, White Plains, N. Y.

TABLE I

The pulmonary diffusing capacity in 21 normal subjects at rest *

Patient	Age	Sex	BSA	f	V_T	$\dot{V}_{O_2}$	R_E	Pa_{CO_2}	$\dot{V}_{CO}$	$\bar{P}_{A_{CO}}$	Pa_{CO}	$V_D/V_T \times 100$	$CO_F \times 100$	D_{LCO}
			M.²	*per min.*	*ml.*	*ml./min.*		*mm. Hg*	*ml./min.*	*mm. Hg*	*mm. Hg*	*%*	*%*	*ml./min./ mm. Hg*
R. L.	17	M	1.75	18.5	390	257	0.83	41	3.05	0.153	0.003	21	60	20.3
J. P.	17	M	1.95	22.5	427	296	0.76	41	3.16	0.181	0.013	35	45	18.8
J. R.	36	M	1.96	18.5	516	310	0.82	41	3.54	0.155	0.003	32	51	23.3
S. B.	59	M	2.10	17.5	594	296	0.83	36	3.32	0.230		33	40	14.4
O. B.	31	M	1.78	9.5	561	222	0.71	41	2.05	0.210	0.018	27	48	10.7
J. P.	40	M	2.09	17.5	508	295	0.77	38	3.03	0.261	0.004	27	44	11.8
C. B.	41	M	1.69	14.5	487	236	0.78	39	2.50	0.213		28	45	11.7
H. S.	35	M	1.91	16.0	548	257	0.97	39	3.00	0.271	0.012	26	46	11.5
E. R.	49	M	1.74	15.5	472	226	0.85	40	2.79	0.208		30	48	13.4
M. O.	28	M	1.98	15.0	530	298	0.72	39	3.22	0.213	0.001	28	50	15.2
T. M.	17	M	1.96	14.5	543	291	0.82	38	3.71	0.159	0.003	21	54	23.7
C. R.	24	M	1.80	14.0	377	189	0.85	41	2.22	0.186	0.024	20	55	13.7
A. A.	48	M	2.01	14.5	484	222	0.78	42	2.55	0.186	0.002	38	47	13.8
M. H.	36	M	1.85	15.0	514	246	0.89	41	2.88	0.236	0.020	29	46	13.3
J. L.	39	M	1.96	18.5	440	238	0.74	36	2.41	0.246	0.047	35	38	12.0
M. I.	30	M	1.68	17.5	392	225	0.79	38	2.63	0.206	0.004	26	50	13.0
E. G.	61	M	1.93	18.0	532	311	0.78	40	3.10	0.221	0.001	39	40	14.0
P. G.	56	M	1.34	22.0	389	202	0.75	42	2.05	0.201	0.009	40	36	10.7
D. O'D.	44	F	1.62	19.0	412	214	0.86	40	2.57	0.280	0.039	35	41	10.7
M. M.	62	F	1.52	20.0	393	266	0.67	43	1.74	0.203	0.019	34	43	9.0
L. D.	55	F	1.60	15.0	366	218	0.74	42	1.49	0.157	0.021	38	44	11.0

* Symbols: BSA = body surface area, square meters; f = respiratory frequency, breaths per minute; V_T = tidal volume, ml. BTPS; $\dot{V}_{O_2}$ = oxygen consumption, ml. per minute STPD; R_E = respiratory exchange ratio, expired air; Pa_{CO_2} = tension of carbon dioxide in arterial blood, mm. Hg; V_{CO} = uptake of carbon monoxide, ml. per minute STPD; $\bar{P}_{A_{CO}}$ = mean alveolar carbon monoxide tension, mm. Hg; Pa_{CO} = tension of carbon monoxide in arterial blood, mm. Hg; $V_D/V_T \times 100$ = ratio of personal dead space (total dead space minus the instrument dead space) to tidal volume, per cent; $CO_F \times 100$ = ratio of the uptake of carbon monoxide to the volume of carbon monoxide inspired, per cent; and D_{LCO} = diffusing capacity of the lung for carbon monoxide, ml. per minute per mm. Hg.

TABLE II

*The respiration, circulation and pulmonary diffusing capacity in normal subjects at rest, during exercise and voluntary hyperpnea**

Patient, Age, Sex, BSA/M.²	State	f	V_T	$\dot{V}_E$	V_{O_2}	R_E	$P_{a_{CO_2}}$	V_{CO}	$\bar{P}_{A_{CO}}$	$P_{a_{CO}}$	V_D/V_T ×100	CO_T ×100	$D_{L_{CO}}$	$\dot{Q}$
		per min.	*ml.*	*L./min.*	*ml./min.*		*mm. Hg*	*ml./min.*	*mm. Hg*	*mm. Hg*	%	%	*ml./min./ mm. Hg*	*L./min.*
Rest and exercise														
H. K.	Rest	18.5	542	8.35	260	0.76	40	2.18	0.257	0.011	26	35	8.9	6.7
24, F	Exer.	23	839	16.05	498	0.89	39	4.78	0.279	0.038	29	36	19.8	8.30
1.68	Exer.	26	945	20.42	636	0.99	43	5.31	0.350	0.072	20	29	19.1	8.96
A. S.	Rest	17	439	6.16	284	0.66	32	2.94	0.226	0.018	19	51	14.1	8.6
28, M	Exer.	34	642	18.01	595	0.88	33	6.22	0.330	0.050	27	36	22.2	12.4
1.65	Exer.	40	768	25.30	706	0.95	32	6.66	0.399	0.099	33	27	22.2	13.8
N. L.	Rest	19	362	5.66	216	0.91	40	2.46	0.192	0.015	22	51	14.0	5.02
26, F	Exer.	29	597	14.20	618	0.89	38.5	4.89	0.344	0.028	20	37	15.5	9.50
1.51	Exer.	30	834	20.60	802	0.96	35	6.10	0.393	0.061	22	31	18.4	11.30
	Rest	17	416	5.83	254	0.88	40	2.49	0.185	0.030	23	49	16.0	6.20
C. M.	Rest	19	349	5.39	202	0.78	37	1.85	0.208	0.013	27	44	10.0	
34, F	Exer.	36	585	17.15	554	0.84	36.5	4.08	0.319	0.055	37	28	15.4	
1.58														
D. E.	Rest	13	827	8.79	320	0.82	31	3.47	0.232	0.024	25	47	16.7	
36, M	Exer.	19.5	1,055	16.82	954	0.87	33	6.01	0.280	0.038	28	41	24.8	
2.20	Exer.	21	1,310	22.50	1,051	0.92	39	7.43	0.337	0.054	23	38	26.3	
Rest, exercise and voluntary hyperpnea†														
A. S.	Rest	12	519	5.07	256	0.74	39.5	2.56	0.191	0.009	22	54	14.1	6.24
24, M	Exer.	21	1,007	17.25	694	1.06	38.5	7.54	0.317	0.032	17	45	26.5	8.47
1.89	Hyper.	20	1,270	20.70	255	1.00	36.5	7.24	0.284	0.050	39	36	30.9	5.67
M. M.	Rest	15.5	506	6.45	230	0.80	33	3.08	0.242	0.005	22	51.3	13.0	6.76
27, F	Exer.	22.0	1,012	18.34	542	1.07	36	6.48	0.335	0.027	31	35.1	21.0	10.04
1.61	Hyper.	20.5	825	15.60	283	0.78	37	5.60	0.368	0.090	31	36.0	20.0	8.09
C. C.	Rest	8	1,045	6.86	254	0.89	35	3.70	0.258	0.023	29	55	15.7	4.38
44, M	Exer.	20	925	15.19	664	0.90	37	5.84	0.312	0.033	30	38	21.0	7.64
1.81	Hyper.	10.5	1,776	15.31	282	0.82	40	5.42	0.298	0.051	35	35	22.0	5.42
	Hyper.	26.0	1,120	23.70	280	0.92	38	6.21	0.382	0.053	46	26	18.0	4.59
L. S.	Rest	10.5	539	4.63	203	0.79	34.5	2.35	0.155	0.006	19	60	15.8	5.34
20, F	Exer.	29.5	905	27.80	704	1.04	35	7.90	0.277	0.027	26	41	31.5	9.38
1.61	Hyper.	22	899	16.20	332	0.51	40	5.92	0.279	0.040	23	42	24.8	6.74
R. G.	Rest	19.5	482	7.80	183	0.80	36	3.72	0.312	0.015	26	49	12.5	
15, M	Exer.	30	611	15.20	591	0.90	38	5.84	0.340	0.025	23	39	18.6	
1.62	Hyper.	15	1,125	14.00	263	1.38	26	5.92	0.313	0.038	23	43	21.6	
J. R.	Rest	12	678	6.55	353	0.71	40	3.03	0.178	0.028	25	45	20.2	
26, M	Exer.	24	1,391	26.90	1,143	0.97	38	8.60	0.361	0.044	20	35	27.1	
2.08	Hyper.	4.3	3,020	10.52	452	0.98	36	5.43	0.206	0.048	17	56	33.7	
	Hyper.	14	3,329	37.50	529	1.77	26	9.00	0.384	0.052	31	26	27.1	
J. J.	Rest	19	356	5.63	251	0.71	40	2.22	0.185	0.028	32	48	14.2	6.4
43, M	Exer.	25	1,077	22.40	934	1.10	40	7.05	0.354	0.047	36	36	23.0	10.74
1.59	Rest	16	436	5.81	245	0.75	40	2.62	0.204	0.046	32	55	16.6	6.8
	Hyper.	25	1,544	31.85	350	0.43	46	7.89	0.340	0.067	32	28	28.9	8.14
E. M.	Rest	17	366	5.17	218	0.75	40	2.04	0.149	0.012	35	47	14.9	6.61
51, M	Exer.	21.5	1,105	19.80	798	1.12	40	5.32	0.264	0.046	42	28	24.4	12.68
1.65	Rest	17.5	380	5.53	240	0.65	40	2.12	0.176	0.056	36	45	17.6	7.50
	Hyper.	22	1,329	24.20	322	0.42	44	7.67	0.351	0.061	22	35	26.6	7.33

* Symbols are the same as in Table I, plus: $\dot{V}_E$ = total ventilation (expired air), L. per minute, STPD and $\dot{Q}$ = pulmonary blood flow, L. per minute.

† During voluntary hyperpnea, Subjects A. S., M. M. and C. C. breathed 3 per cent CO_2 in air; Subjects J. J., E. M., B. K., J. I. and L. S. breathed 5 per cent CO_2 in air; and Subjects R. G. and J. R. breathed ambient air.

TABLE II—*Continued*

Patient, Age, Sex, BSA/M.[2]	State	f	V_T	$\dot{V}_E$	$\dot{V}_{O_2}$	R_E	P_{aCO_2}	$\dot{V}_{CO}$	$\bar{P}_{ACO}$	P_{aCO}	V_D/V_T ×100	CO_F ×100	D_{LCO}	$\dot{Q}$
		per min.	*ml.*	*L./min.*	*ml./min.*		*mm. Hg*	*ml./min.*	*mm. Hg*	*mm. Hg*	%	%	*ml./min./ mm. Hg*	*L./min.*
B. K.	Rest	14	554	6.37	272	0.82	44	2.41	0.190	0.017	32	45	13.9	6.80
41, M	Exer.	28	937	21.58	1,104	1.06	47	7.28	0.306	0.026	25	38	26.1	16.20
1.79	Rest	14	560	6.44	292	0.78	46	2.51	0.180	0.036	32	46	17.4	6.80
	Hyper.	24	1,234	24.00	291	0.80	51	8.57	0.280	0.063	27	40	39.6	7.67
J. I.	Rest	21	370	6.38	259	0.72	43	2.42	0.270	0.015	27	43	9.5	6.8
26, M	Exer.	31	683	17.35	692	0.92	44	5.16	0.331	0.029	35	32	17.1	10.2
1.77	Hyper.	32	720	18.90	370	0.67	42	5.20	0.293	0.056	46	29	21.9	7.88

a unilateral pulmonary lesion (Table III), D_{LCO} and pulmonary blood flow were measured for each lung separately during normal flow and during partial obstruction to flow through one pulmonary artery.

The measurement of blood flow through each lung separately was done as previously described (12). In brief, the techniques included: *1*) bronchospirometry for the administration of different inspired mixtures to each lung, as well as the collection of expired gas from each lung separately; *2*) cardiac catheterization with a triple lumen catheter to allow sampling and injection proximal and distal to the occlusive balloon; and *3*) arterial cannulation.

The rate of blood flow through each lung was changed by inflating the balloon in one pulmonary artery. Blood flow was thus diminished to one lung and correspondingly increased in the opposite one.

The lung with the occluded pulmonary artery breathed 25 per cent O_2, while the contralateral lung breathed 21 per cent O_2. Following a 15 minute period of equilibration, each lung continued to breathe its own inspired mixture plus 0.1 per cent CO for an additional four minutes. Blood and gas samples for the calculation of O_2 uptake, CO uptake, cardiac output and arterial blood pCO_2 were collected during the last two minutes of the four minute period.

By this protocol, *total* blood flow may be calculated by dividing O_2 uptake of both lungs by the corresponding arteriovenous difference for O_2. The blood flow through the lung receiving 25 per cent O_2 is calculated from its O_2 uptake and the arteriovenous O_2 difference across that lung, on the assumption that the pulmonary venous blood from that lung is 98 per cent saturated. The flow through the contralateral lung is measured as the difference between the total blood flow and the flow through the lung receiving the 25 per cent O_2. For these measurements, it is assumed on the basis of previous studies in this laboratory that during occlusion of one pulmonary artery, there is no significant bronchial collateral circulation (13) to the compromised side.

For the calculation of the D_{LCO} for each lung separately, during both the control and test periods, separate estimates of pulmonary dead space were required: During the control period, dead space was calculated for each lung by the Bohr formula on the assumption that the arterial tension of carbon dioxide is equal to the alveolar tension of carbon dioxide. During the period of partial occlusion, the calculation was more indirect: a total dead space for both lungs was calculated by the Bohr relationship using the tidal volume, the fraction of CO_2 in expired gas from both lungs and the arterial pCO_2. The dead space of the lung through which flow was increased was similarly calculated from the arterial pCO_2 and the expired fraction of CO_2 from that lung. The dead space of the remaining lung was calculated as the difference between these two. Where occlusion of a pulmonary artery was complete, as in J. M., no estimate of physiologic dead space in that lung was possible; in this case, the control dead space was used in the calculations of D_{LCO}.

CALCULATIONS

In addition to the measurement of the diffusing capacity of the lung for carbon monoxide (D_{LCO}), the uptake of CO by the lung per minute ($\dot{V}_{CO}$), and the ratio of the uptake of CO to the volume of CO inspired (CO_F) have been used as indices of the diffusing capacity.

The pulmonary diffusing capacity is calculated according to the following expression of Fick's law of diffusion:

$$D_{LCO} = \frac{\dot{V}_{CO}}{\bar{P}_{ACO} - \bar{P}_{CCO}},$$

where

D_{LCO} = diffusing capacity of the lung for carbon monoxide in ml. per minute per mm. Hg,

$\dot{V}_{CO}$ = uptake of carbon monoxide, ml. per minute,

$\bar{P}_{ACO}$ = mean alveolar carbon monoxide tension, mm. Hg and

$\bar{P}_{CCO}$ = mean tension of carbon monoxide in pulmonary capillary blood, mm. Hg.

The uptake of CO is calculated from an analysis of inspired and expired fractions of CO using a nitrogen correction for metabolic gas exchange:

$$\dot{V}_{CO} = \dot{V}_E \left(\frac{F_{EN_2}}{F_{IN_2}} F_{ICO} - F_{ECO} \right),$$

where

$\dot{V}_E$ = total minute ventilation, L. per minute, STPD,

F_{EN_2} = fraction of nitrogen in expired gas,

F_{IN_2} = fraction of nitrogen in inspired gas,

F_{ICO} = fraction of carbon monoxide in inspired gas and

F_{ECO} = fraction of carbon monoxide in expired gas.

TABLE III

The ventilation, circulation and pulmonary diffusing capacity of each lung separately *

Patients, Age, Diagnosis, BSA/M.²	State†	Lung‡	$\dot{V}_E$	$\dot{V}_{O_2}$	R_E each lung	R_E both lungs	V_D/V_T ×100	$\dot{V}_{CO}$	CO_F ×100	$\bar{P}_{ACO} - \bar{P}_{aCO}$	D_{LCO}	$\dot{Q}$	$\frac{\Delta D_{LCO}}{D_{LCO}}$‖ ×100	$\frac{\Delta \dot{V}_{CO}}{\dot{V}_{CO}}$‖ ×100	$\frac{\Delta \dot{Q}}{\dot{Q}}$‖ ×100
			L./min.	*ml./min.*			%	*ml./min.*	%	*mm. Hg*	*ml./min./ mm. Hg*	*L./min.*	%	%	%
C. L.	Control	R	4.01	128	0.61	0.72	43	1.20	30	0.350	3.4	5.52			
57, 1.67,		L	5.00	136	0.82		39	1.32	28	0.313	4.2	3.58			
Infiltrates RLL; RML	LPA occluded	R	4.00	150	0.69	0.83	32	1.16	29	0.372	3.1	7.96	− 6	− 3	+ 44
		L	5.11	86	1.08		51	1.09	22	0.290	3.8	2.20	−10	−17	− 38
	Control	R	4.87	123	0.91	0.98	38	1.12	23	0.368	3.0	6.06			
		L	5.37	119	1.06		39	1.23	24	0.316	3.9	2.90			
	LPA occluded	R	3.95	153	0.66	0.77	29	1.13	29	0.383	2.9	6.58	− 3	+ 1	+ 9
		L	4.74	89	0.97		48	0.97	21	0.311	3.1	2.05	−20	−29	− 21
J. C.	Control	R	6.83	189	0.71	0.71	46	2.67	39	0.238	11.2	4.02			
54, 1.71,		L	3.91	163	0.71		25	2.41	62	0.250	9.7	2.75			
Bronchiectasis RML; RLL	RPA occluded	R	5.99	32	1.53	0.69	75	1.40	23	0.370	3.8	0.43	−66	−48	− 89
		L	3.51	228	0.58		20	2.16	63	0.263	8.2	9.18	−15	−10	+230
	RPA occluded	R	6.81	12	3.00	0.73	77	1.04	15	0.459	2.3	0.21	−80	−61	− 94
		L	3.96	235	0.61		24	2.12	55	0.260	8.2	7.49	−15	−12	+170
J. Mc.	Control	R	5.38	169	0.84	0.87	39	2.45	46	0.222	11.0	5.11			
55, 1.95,		L	4.68	75	0.94		61	0.94	20	0.180	5.2	1.66			
Carcinoma left hilum	LPA occluded	R	8.57	251	0.94		36	2.75	32	0.287	9.6	7.18	−15	+12	+ 40
		L	5.20	0			55¶	0.43	8	0.420	1.0	0	−80	−53	−100
		R	8.24	241	0.94		35	2.76	33	0.280	9.9	6.9	−13	+13	+ 35
		L	5.79	0			50¶	0.50	8	0.380	1.3	0	−73	−47	−100
A. J.	Control	R	6.50	150	0.91	1.10	42	1.70	26	0.300	5.7	2.98			
51, 1.74,		L	7.88	124	1.33		44	1.86	24	0.298	6.2	2.06			
Bilateral apical infiltration	RPA occluded	R	4.92	89	1.08	0.97	46	1.23	25	0.328	3.7	1.97	−35	−28	− 34
		L	6.50	183	0.92		30	1.91	30	0.351	5.4	4.07	−13	+ 3	+ 98
E. W.	Control	R	6.54	163	0.88	0.95	45	1.63	26	0.300	5.5	3.80			
48, 1.85,		L	4.31	110	1.05		29	1.40	27	0.282	5.0	3.03			
Normal lungs	RPA occluded	R	4.90	89	0.82	0.73	62	0.82	17	0.219	3.7	1.6	−15	−50	− 58
		L	3.40	148	0.68		31	1.43	43	0.246	5.6	4.48	+12	+ 2	+ 48
	RPA occluded	R	4.52	36	2.01	0.92	59	0.84	19	0.229	3.7	0.74	−31	−48	− 80
		L	3.90	167	0.68		33	1.41	37	0.250	5.6	4.91	+ 2	+ 1	+ 62
T. G.	Control	R	4.46	87	0.84	0.72	59	0.95	22	0.226	4.2**	3.73			
52, 1.80,		L	4.97	214	0.66		38	1.42	30	0.374	3.8**	4.20			
Carcinoma right lung	RPA occluded	R	5.65	48	0.95	0.63	76	0.66	12	0.183	3.6	2.91	−14	−31	− 22
		L	5.50	288	0.57		34	1.49	28	0.383	3.9	4.24	+ 3	+ 5	+ 1

* Symbols as in Table I.
† The lung with the occluded pulmonary artery breathed 25 per cent O_2 while the contralateral lung breathed 21 per cent O_2. The same mixtures, plus 0.1 per cent CO, were used to measure D_{LCO}.
‡ R refers to right lung; L to left lung.
‖ Represents $\frac{\text{final value} - \text{initial value}}{\text{initial value}}$.
¶ Same dead space as in control period.
** The D_{LCO} of both lungs, measured two days later without bronchospirometry, was 8.2 ml. per minute per mm. Hg.

The mean alveolar tension of CO is calculated from the Bohr dead space assuming that $V_{D_{CO_2}} = V_{D_{CO}}$, so that:

$$\bar{P}_{A_{CO}} = \frac{V_T(P_{E_{CO}}) - V_{D_{CO_2}}(P_{I_{CO}})}{V_T - V_{D_{CO_2}}},$$

where

$V_{D_{CO_2}}$ = dead space for carbon dioxide based on arterial blood pCO_2,

$V_{D_{CO}}$ = dead space for carbon monoxide and

V_T = tidal volume, BTPS.

The fraction of CO taken up from inspired gas was calculated according to Filley, MacIntosh and Wright (6) as follows:

$$CO_F = \frac{V_{CO}}{V_E\left(\frac{F_{E_{N_2}}}{F_{I_{N_2}}} F_{I_{CO}}\right)} - V_D \text{ (instrument) (f)} + (F_{I_{CO}} - F_{A_{CO}}),$$

where

CO_F = ratio of the uptake of carbon monoxide to the volume of carbon monoxide inspired, per cent,

$F_{A_{CO}}$ = fraction of carbon monoxide in alveolar gas and

f = respiratory frequency, breaths per minute.

The arterial tension of CO was calculated from the Haldane relationship (8):

$$P_{a_{CO}} = \frac{P_{a_{O_2}} \cdot (COHb)}{210 \cdot (O_2Hb)},$$

where

$P_{a_{CO}}$ = tension of carbon monoxide in arterial blood, mm. Hg,

$P_{a_{O_2}}$ = tension of oxygen in arterial blood, mm. Hg,

(COHb) = content of carboxyhemoglobin in arterial blood, volumes per cent,

(O_2Hb) = content of oxyhemoglobin in arterial blood, volumes per cent and

210 = relative affinity constant for carbon monoxide and oxygen in blood.

RESULTS

Rest and exercise

The $D_{L_{CO}}$ was measured at rest in 36 normal subjects. These individual measurements and the data from which they were derived appear in Tables I and II. The average resting $D_{L_{CO}}$ for this entire group was 14.1 ml. per minute per mm. Hg.

The corresponding values for patients with rheumatic heart disease appear in Table IV. The average resting $D_{L_{CO}}$ for this group of subjects is 14.6 ml. per minute per mm. Hg, which is not significantly different ($p > 0.05$) from the normal subjects.

An analysis of the resting $D_{L_{CO}}$ according to sex, for both normal subjects and patients with rheumatic heart disease, is included in Table V. It may be seen that the mean resting value for the normal males (14.8) is higher than the mean resting value for normal females (11.5). This difference is only of suggestive statistical significance ($p < 0.05$).

It is of interest, that the mean $D_{L_{CO}}$ of the three male subjects in the rheumatic group is higher than the value in the normal subjects. However, the number of subjects is obviously too few for statistical comparison.

The diffusing capacity during exercise and the data from which they were derived are shown in Table II for normal subjects and in Table IV for subjects with rheumatic heart disease. It may be seen that each subject experienced an increase in diffusing capacity during exercise.

As is shown in Table V, the mean $D_{L_{CO}}$ during exercise in normal subjects is slightly greater (22.1 ml. per minute per mm. Hg) than the mean exercise $D_{L_{CO}}$ in subjects with rheumatic heart disease (20 ml. per minute per mm. Hg). However, Table V also indicates that the normal subjects achieved a higher level of exercise (mean O_2 uptake of 430 ml. per minute per $M.^2$) than did subjects with rheumatic heart disease (mean O_2 uptake of 320 ml. per minute per $M.^2$). When this difference in level of exercise is taken into account by expressing the increase in $D_{L_{CO}}$ as per 100 ml. increase in oxygen uptake, both groups demonstrate an average increase in $D_{L_{CO}}$ of 1.9 ml. per minute per mm. Hg per 100 ml. increase in O_2 uptake.

In Figure 1, the $D_{L_{CO}}$ is related to minute ventilation and to pulmonary blood flow for both groups of subjects at rest and during exercise. It may be seen, in Figure 1A, that there is a good statistical correlation ($p < 0.001$) between $D_{L_{CO}}$ and the minute ventilation, at rest and during exercise; on the other hand, as seen in Figure 1B, the correlation between $D_{L_{CO}}$ and pulmonary blood flow is poorer ($p = 0.01$). Figure 1 emphasizes that during exercise, $D_{L_{CO}}$ increases in subjects with mitral stenosis in the face of abnormally low increments in pulmonary blood flow.

Figure 2 substitutes CO uptake for $D_{L_{CO}}$ and shows a similar type of relationship with minute ventilation and pulmonary blood flow.

TABLE IV

The ventilation, circulation and pulmonary diffusing capacity in patients with rheumatic heart disease at rest, during exercise and voluntary hyperpnea *

Patients, Age, Sex, BSA/M.²	State	f	V_T	$\dot{V}_{O_2}$	R_E	P_{ACO_2}	$\dot{V}_{CO}$	$\bar{P}_{ACO}$	P_{ACO}	D_{LCO}	CO_E X100	V_D/V_T X100	$\dot{Q}$	P.A. pressure S	P.A. pressure D	P.A. pressure M
		per min.	*ml.*	*ml./min.*		*mm. Hg*	*ml./min.*	*mm. Hg*	*mm. Hg*	*ml./min./ mm. Hg*	%	%	*L./min.*	*mm. Hg*		
							Rest and exercise									
F. R.	Rest	10	597	277	0.72	43	2.36	0.096	0.009	27.1	59	28	3.60	34	19	29
40, M	Exer.	25.5	1,444	1,023	1.02	42	10.56	0.286	0.031	41.5	39	38	6.80	76	50	60
1.85																
C. M.	Rest	17	460	170	0.91	37	2.23	0.210	0.013	11.4	45	30	3.54	42	22	31
51, F	Exer.	40	788	634	1.01	30	5.90	0.359	0.045	18.8	26	36	8.93	55	32	43
1.51																
P. B.	Rest	16.5	514	145	0.79	37	2.07	0.201	0.011	10.9	45	25	5.00	40	16	27
37, F	Exer.	28.0	596	417	0.96	36	4.25	0.230	0.041	22.5	41	35	7.74	65	28	41
1.37																
F. L.	Rest	25.5	457	216	0.72	32	2.68	0.181	0.019	16.6	49	25	4.80	28	15	18
31, F	Exer.	26	602	300	0.87	38	3.18	0.221	0.038	17.3	33	33	5.10	37	20	29
1.23																
L. D.	Rest	15.5	583	275	0.75	31	2.59	0.306	0.021	9.1	38	26	5.85	61	25	37
43, F	Exer.	30	964	648	1.06	30	5.33	0.454	0.044	13.0	24	26	5.89	97	55	74
1.57																
G. A.	Rest	16	529	235	0.84	31	3.29	0.178	0.015	20.2	54	23	4.52	40	18	26
56, F	Exer.	25	740	480	0.90	33	5.31	0.228	0.031	27.0	38	33	5.16	72	44	58
1.66																
M. I.	Rest	20	385	231	0.83	40.5	2.42	0.238	0.011	10.7	43	31	5.02	67	34	45
31, F	Exer.	29.5	556	423	0.97	40	4.22	0.343	0.029	13.4	33	35	6.32	110	47	67
1.59																
H. K.	Rest	10.0	653	270	0.77	42	2.79	0.178	0.011	16.8	53	29	4.66	60	24	38
40, M	Exer.	20.0	760	496	0.89	41.5	5.28	0.239	0.029	25.1	45	32	5.76	84	32	52
1.93																
R. D.	Rest	13.5	480	267	0.74	39	2.47	0.211	0.021	13.0	50	25	4.31	41	18	28
34, M	Exer.	22	881	422	0.97	40	5.40	0.330	0.046	19.0	35	22	4.26	75	38	55
1.61																
							Rest, exercise and voluntary hyperpnea †									
S. C.	Rest	14	602	202	0.75	31.5	2.40	0.275	0.016	9.3	37	35	4.04	61	23	36
51, F	Exer.	18	839	454	0.93	31	4.77	0.373	0.027	13.8	25	43	5.82	105	48	70
1.47	Hyper.	24	713	233	0.75	42	3.72	0.324	0.040	13.1	27	46	4.68	61	21	36
V. W.	Rest	13.5	385	172	0.74	35	1.97	0.184	0.003	10.9	52	23	3.19	60	31	41
34, F	Exer.	22	567	357	0.93	36	3.99	0.292	0.019	14.6	41	25	3.40	114	66	86
1.36	Hyper.	21	638	‡		35	3.63	0.253	0.049	17.7	35	24		72	36	47‖
L. L.	Rest	11	417	215	0.62	40	2.14	0.099	0.004	22.6	63	23	5.52	40	23	33
33, F	Exer.	24	752	570	1.00	41	6.29	0.251	0.021	27.2	42	26	7.70	82	43	60
1.63	Hyper.	20	1,420	250	0.95	35	6.88	0.254	0.028	30.4	29	50	6.24	33	15	24
I. R.	Rest	16	318	185	0.73	43	1.72	0.177	0.012	10.4	50	31	4.52	20	5	13
32, F	Exer.	25.5	484	275	1.00	40	3.16	0.278	0.041	13.3	35	40	4.67	24	8	16
1.43	Hyper.	18	750	220	0.50	42	4.13	0.246	0.052	21.3	41	37	4.68	19	8	12

* Symbols are the same as in Table I, plus P.A. pressure, SDM = pressure in the pulmonary artery; systolic, diastolic and mean, mm. Hg.
† During voluntary hyperpnea, Subjects S. C., V. W. and L. L. breathed 3 per cent CO_2 in air; Subject I. R. breathed 5 per cent CO_2 in air.
‡ No gas analyses; V_D assumed.
‖ Pressure unchanged from level just prior to voluntary hyperpnea.

TABLE V

*The diffusing capacity and fractional uptake of carbon monoxide at rest and during moderate exercise**

Subjects Sex	No.	Age	State	$\dot{V}_{O_2}$	$D_{L_{CO}}$	$CO_F \times 100$
		yrs.		*ml./min./M.²*	*ml./min./mm. Hg*	%
				Normal subjects		
Males	28	(14–61)	Rest	141 (113–172)	14.8 (9.5–23.7) ± 3.48	47 (36–60) ±4.9
Females	8	(20–62)		143 (126–175)	11.5 (9.0–15.8) ± 2.28	45 (34–60) ±8.2
Males	10	(14–44)	Exercise	450 (360–617)	23.3 (17.4–27.1) ± 4.10	36 (27–45) ±4.8
Females	5	(20–44)		391 (296–531)	20.1 (16.0–31.5) ± 5.80	34 (28–41) ±4.0
				Rheumatic heart disease		
Males	3	(34–40)	Rest	151 (140–165)	19.? (14.0–27.1) ± 5.6	54 (50–59) ±3.3
Females	10	(31–51)		139 (112–176)	13.2 (9.1–22.5) ± 3.3	48 (37–63) ±7.4
Males	3	(34–40)	Exercise	357 (262–552)	27.9 (16.8–41.6) ±10.3	40 (35–45) ±4.7
Females	10	(31–57)		305 (193–420)	17.5 (13.0–27.4) ± 4.4	34 (24–42) ±6.5

* Symbols as in Table I. Mean values are followed by range in parentheses; figures after range indicate standard deviation.

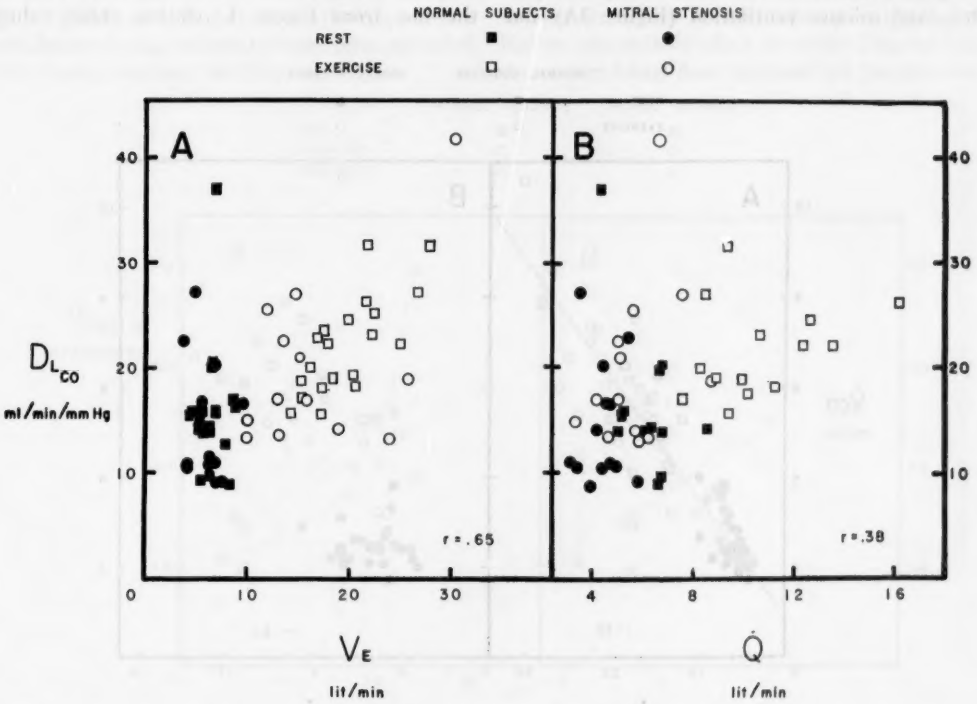

FIG. 1. THE EFFECT OF MINUTE VENTILATION ($\dot{V}_E$) AND PULMONARY BLOOD FLOW ($\dot{Q}$) ON THE DIFFUSING CAPACITY OF THE LUNG FOR CARBON MONOXIDE ($D_{L_{CO}}$) IN NORMAL SUBJECTS AND IN PATIENTS WITH MITRAL STENOSIS, AT REST AND DURING EXERCISE

Although pulmonary blood flow increased only slightly during exercise in the patients with mitral stenosis, the $D_{L_{CO}}$ increased normally (Table V).

Finally, as may be seen from Table V, the amount of CO removed from inspired air averaged 45 per cent in normal subjects at rest and decreased to 35 per cent during exercise. These mean values for patients with mitral stenosis are not significantly different ($p > 0.05$).

Voluntary hyperpnea

The data concerning minute ventilation, pulmonary blood flow and diffusing capacity during voluntary hyperpnea are listed in Tables II and IV. In all but three instances (M. M., L. S. and R. G.), the minute ventilation during voluntary hyperpnea was the same, or greater, than that during exercise. It may be seen that this deliberate augmentation of ventilation up to levels of 32 L. per minute was associated with increases in cardiac output of only 20 per cent above the resting levels.

Figure 3 illustrates the relationship between D_{LCO} and minute ventilation (Figure 3A) and D_{LCO} and pulmonary blood flow (Figure 3B) in four normal subjects and one subject with mitral stenosis at rest, during exercise and during voluntary hyperpnea. It may be seen from Figure 3A that as ventilation is increased, either by voluntary hyperpnea or exercise, the diffusing capacity also increases. However, as may be seen in Figure 3B, the increases in D_{LCO} during voluntary hyperpnea are independent of increases in pulmonary blood flow since: *1)* In the rheumatic subject, the increases in D_{LCO} were unaccompanied by changes in blood flow either during voluntary hyperpnea or exercise, and *2)* in the normal subjects, the largest increments in D_{LCO} occur during voluntary hyperpnea when blood flow was consistently less than during exercise.

In Figure 4A, minute ventilation is plotted against the uptake of carbon monoxide for the subjects of Figure 3. For the sake of reference, the line from Figure 1 relating these values

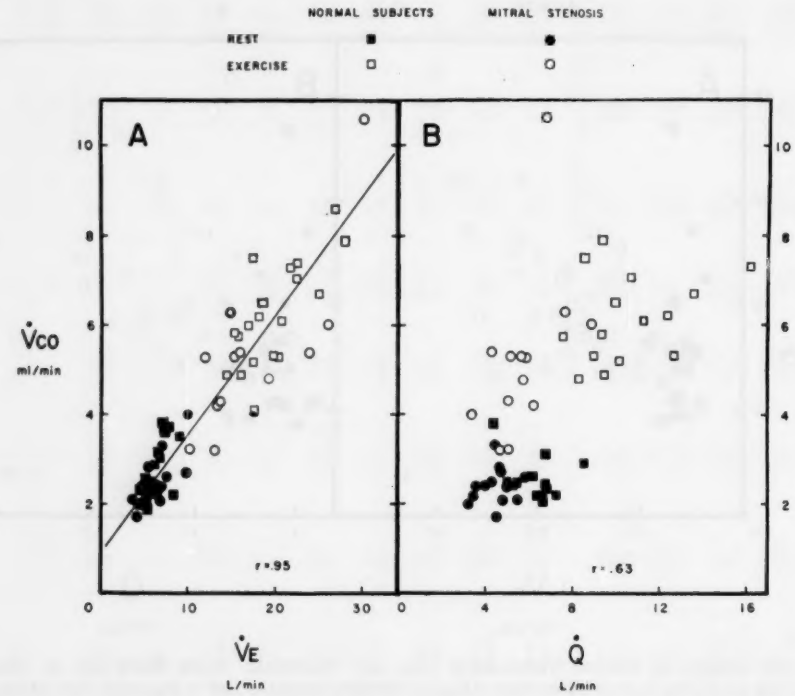

FIG. 2. THE EFFECT OF MINUTE VENTILATION ($\dot{V}_E$) AND PULMONARY BLOOD FLOW (Q) ON THE UPTAKE OF CARBON MONOXIDE ($\dot{V}_{CO}$) IN NORMAL SUBJECTS AND IN PATIENTS WITH MITRAL STENOSIS AT REST AND EXERCISE

during rest and exercise is also included. It may be seen that the relationship between the CO uptake and ventilation during voluntary hyperpnea is the same as that during exercise. As in the case of D_{LCO}, Figure 4B illustrates that these increases in $\dot{V}_{CO}$ during voluntary hyperpnea are also independent of changes in pulmonary blood flow.

The values for the fraction of CO removed from inspired air during voluntary hyperpnea are also included in Tables II and IV. It may be seen that the fraction of CO removed from inspired gas (CO_F) is, in most instances, reciprocally related to the level of ventilation. Thus, when the level of ventilation during voluntary hyperpnea exceeded that during exercise, the CO_F was less than that during exercise. Conversely, when the level of ventilation during hyperpnea was less than that during exercise, the CO_F was higher. However, in three subjects (J. J., E. M. and B. K.), even though minute ventilation during voluntary hyperpnea exceeded that during exercise, the CO_F was higher.

In order to assess the effect of a change in ventilatory pattern on D_{LCO}, the respiratory frequency and tidal volume were varied in two subjects, C. C. and J. R., during two successive periods of voluntary hyperpnea. In both subjects, D_{LCO}, $\dot{V}_{CO}$ and CO_F were higher during breathing patterns of slow frequency and large tidal volume.

Unilateral occlusion of one pulmonary artery

In Table III are listed the data for the calculation of D_{LCO}, $\dot{V}_{CO}$ and pulmonary blood flow ($\dot{Q}$) for each lung separately. These data are also the basis for Figure 5.

The values for oxygen uptake and pulmonary blood flow in Table III indicate that different degrees of occlusion of a pulmonary artery were accomplished in different subjects. Despite these changes in blood flow, minute ventilation in each lung remained relatively unaffected. As may be seen in Figure 5, the increases in blood flow had no appreciable affect on either D_{LCO} or V_{CO}, even when blood flow increased by 230 per cent.

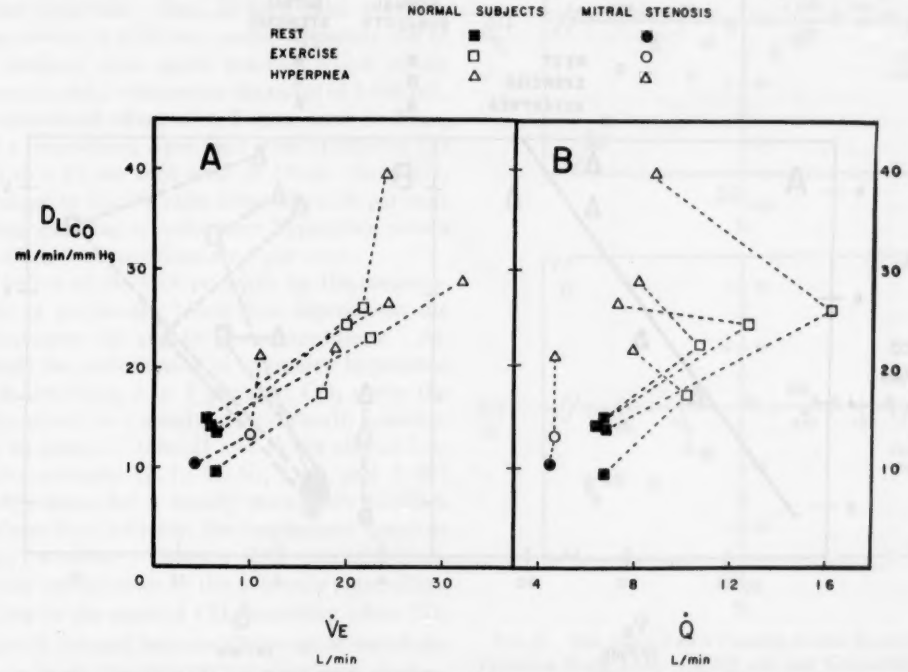

FIG. 3. THE EFFECT OF MINUTE VENTILATION ($\dot{V}_E$) AND PULMONARY BLOOD FLOW (Q) ON THE DIFFUSING CAPACITY OF THE LUNG FOR CARBON MONOXIDE (D_{LCO}) DURING REST, EXERCISE AND VOLUNTARY HYPERPNEA IN FOUR NORMAL SUBJECTS AND ONE PATIENT WITH MITRAL STENOSIS

Only when blood flow was severely curtailed, *i.e.*, to less than 50 per cent of the control value, did a decrease in D_{LCO} and $\dot{V}_{CO}$ become apparent.

Although the fraction of CO removed from inspired air tended to parallel the change in D_{LCO}, individual results are difficult to assess because of unavoidable changes in ventilation between the control and the test periods.

Relation between pulmonary artery pressure and D_{LCO}

In the normal subjects, pulmonary artery pressures averaged 18/7 mm. Hg, with a mean of 12 at rest and increased to 25/11, with a mean of 16 during exercise. In the patients with mitral stenosis (Table IV) pulmonary artery pressure was abnormally high at rest, averaging 46/21 mm. Hg, with a mean of 31; during exercise, the average pulmonary artery pressure rose to 76/39 mm. Hg, with a mean of 50. Both groups of subjects failed to show any change in pulmonary artery pressure during voluntary hyperpnea.

No correlation was demonstrable between the level of pulmonary artery pressure and the D_{LCO} in either group of subjects, either at rest or during exercise.

Effect of correction of tension of CO in arterial blood (Pa_{CO}) on D_{LCO}

As expected, the tension of CO in arterial blood increased with the time of exposure to 0.1 per cent CO (14). The effect of this increase in Pa_{CO} on calculated D_{LCO} is illustrated in Figure 6. It may be seen that when Pa_{CO} is taken into account in the calculation of D_{LCO}, after six minutes of exposure at rest, the D_{LCO} increases by 5 to 10 per cent. The exercise D_{LCO} after 12 minutes of exposure increased by 10 to 15 per cent, and after 18 minutes of exposure D_{LCO} increased by 20 to 30 per cent.

Sources of error in methods

As may be seen from the equations above, and from the data in Tables I through V, the calcu-

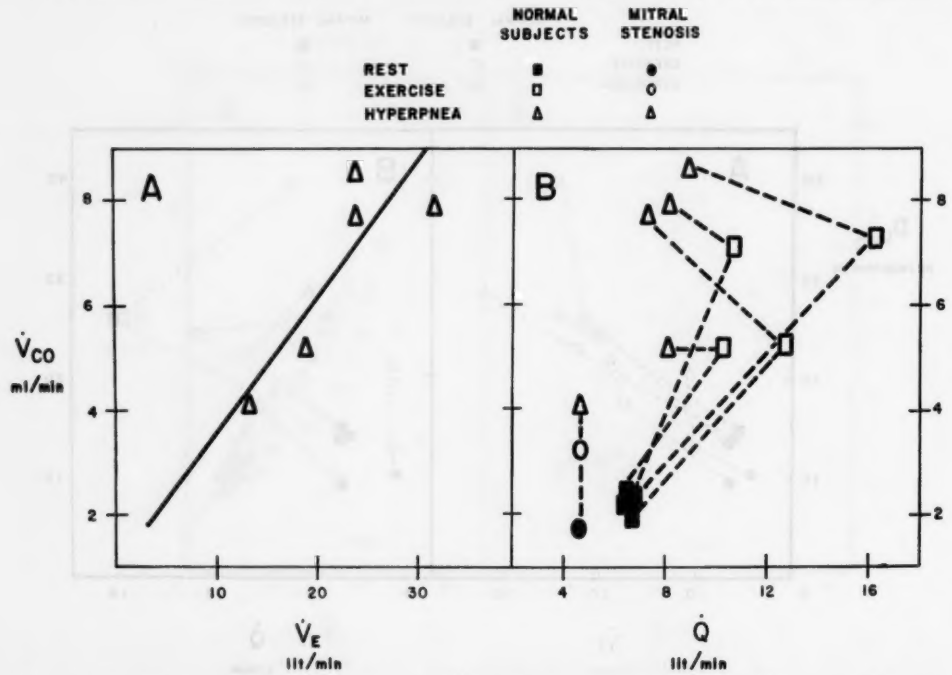

FIG. 4. THE EFFECT OF MINUTE VENTILATION ($\dot{V}_E$) AND PULMONARY BLOOD FLOW ($\dot{Q}$) ON THE UPTAKE OF CARBON MONOXIDE ($\dot{V}_{CO}$) IN THE SUBJECTS OF FIGURE 3 DURING REST, EXERCISE AND VOLUNTARY HYPERPNEA
The diagonal line in Figure 4A is derived from the data of Figure 1A by the method of least squares.

lation of D_{LCO} by the steady state method affords ample opportunity for compounding errors. As indicated by Filley, MacIntosh and Wright (6), the major sources of error are *1*) the measurement of arterial pCO_2 by virtue of its effect on the calculation of the dead space and *2*) the measurement of the expired fraction of carbon monoxide. By the nature of these errors, the estimation of D_{LCO} by the steady state method is more accurate for high, rather than low, tidal volumes. Thus, at rest, where tidal volumes are small, the effect of the dead space volume on the calculation of D_{LCO} is relatively large. An error of 2 mm. in the measurement of arterial pCO_2 may lead to an error of ± 10 per cent in the D_{LCO} by its effect on the dead space measurement. A concordant error in determination of expired CO of 2 per cent of full scale will increase the error in D_{LCO} to ± 25 per cent.

When tidal volumes are large, as during exercise or during voluntary hyperpnea, the effect of changes in dead space volume on the calculation of alveolar pCO are less, so that errors from this source diminish. Also, at high tidal volumes, gross errors in pCO_2 are readily apparent due to the unlikely dead space volumes which result. Assuming tidal volumes in the range of 2,000 ml., the combined effect of a 2 mm. error in Pa_{CO_2} and a concordant 2 per cent error in expired CO lead to a 12 per cent error in D_{LCO}. Similarly, a change in V_D/V_T ratio from 12 to 39 per cent during exercise or voluntary hyperpnea varies the D_{LCO} by approximately 9 per cent.

The use of the Fick principle for the measurement of pulmonary blood flow depends on the maintenance of steady state conditions. Although the performance of voluntary hyperpnea while breathing 3 or 5 per cent CO_2 made the achievement of a steady state difficult, nonetheless, as seen in Tables II and IV, in all but four of the subjects (J. J., N. N., L. S. and I. R.) strict criteria for a steady state were fulfilled. In these four subjects, the respiratory quotient (R.Q.) was low. This low R.Q. presumably reflects a continuation of the unsteady state which obtains at the start of CO_2 breathing when CO_2 output is reduced because of storage of metabolic CO_2 in body tissues (15). Under such circumstances, the O_2 uptake by the lungs calculated from the fractions of O_2 and CO_2 in expired gas is artificially high. The high O_2 uptake leads to an elevation of cardiac output calculated by the Fick principle. Such considerations suggest that in these subjects with a low R.Q. during voluntary hyperpnea, the *actual* increase in cardiac output was even less than indicated in Table V.

DISCUSSION

These different types of experiments are in accord in demonstrating a marked effect of minute ventilation on D_{LCO}. By way of contrast, they indicate that D_{LCO} is little affected by a change in pulmonary blood flow until flow is reduced well below 50 per cent of normal. They also offer some basis for speculation concerning the effect of the pulmonary blood volume on the pulmonary diffusing capacity.

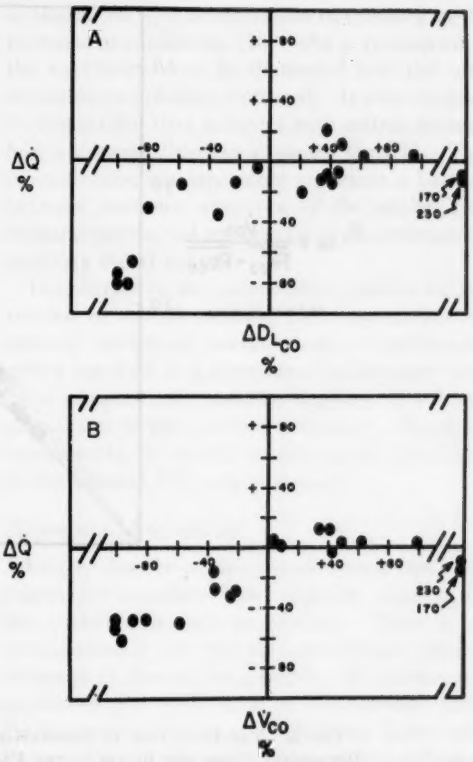

FIG. 5. THE EFFECT OF A CHANGE IN THE BLOOD FLOW THROUGH EACH LUNG (% $\Delta\dot{Q}$) ON THE CORRESPONDING DIFFUSING CAPACITY FOR CARBON MONOXIDE (% ΔD_{LCO}) (UPPER FIGURE) AND ON THE UPTAKE OF CO (% $\Delta\dot{V}_{CO}$) (LOWER FIGURE)

Ventilation

It is generally believed that during quiet breathing only a fraction of the alveolar surface is used for diffusion. During exercise, as minute ventilation increases, it is likely that the alveolar-capillary membrane is stretched and the area used for diffusion increases.

Previous indirect estimates of D_{LCO}, such as the fractional uptake of CO (16), as well as direct measurements by the single breath technique (3, 17), suggest that the volume of gas in the lung influences the area available for alveolar-capillary gas exchange. Some support for this point of view is brought forth in the present study, where an enlarged *mean* alveolar volume, presumably accomplished by slow deep breathing was associated with values of D_{LCO} greater than with usual breathing patterns.

It is pertinent to note that the relationship between the volume of alveolar gas and the D_{LCO} may involve several less evident physiological adaptations: *1*) a redistribution of blood within the lung so as to preserve CO gradients for diffusion, and *2*) absolute increases in both blood flow (18, 19) and volume (20) incident to respiratory maneuvers which involve deep or prolonged inspirations.

Although these hidden mechanisms cannot be assessed, it is nonetheless clear that methods for determining D_{LCO} which involve increases in the mean alveolar gas volume such as the single breath technique of Forster, Fowler, Bates and

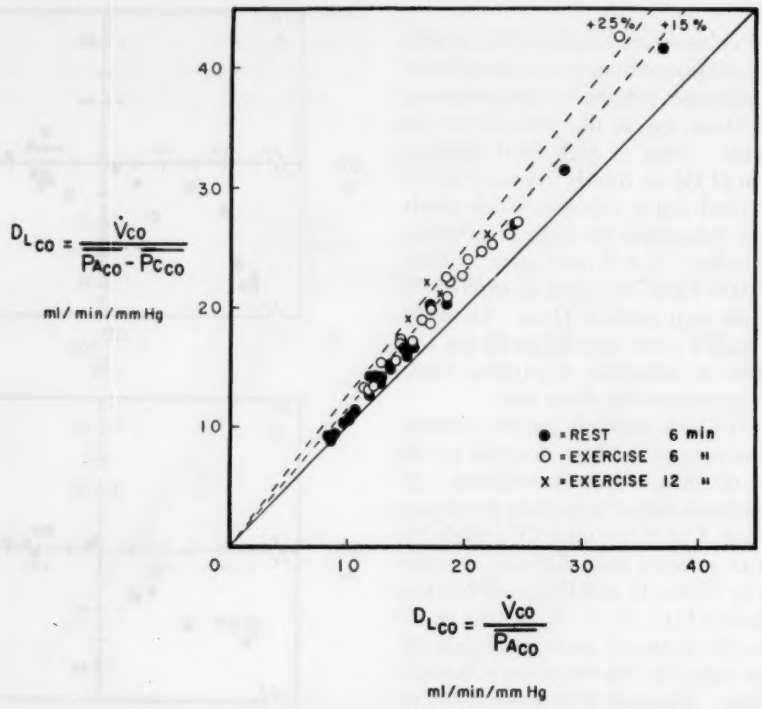

FIG. 6. THE EFFECT OF AN ESTIMATED "BACK PRESSURE" OF CARBON MONOXIDE IN PULMONARY CAPILLARY BLOOD ON THE CALCULATED VALUE FOR DIFFUSING CAPACITY OF THE LUNG

The values on the ordinate are calculated on the assumption that the mean tension of CO in arterial blood equals the mean tension of CO in the pulmonary capillary. The values for the abscissa are calculated on the assumption that the mean tension of CO in the pulmonary capillary is zero.

Van Lingen (21) and the rebreathing technique of Kruhøffer (22) should yield larger values for D_{LCO} at rest than do methods utilizing normal breathing patterns. These differences have been observed (23).

Rate of pulmonary blood flow

On the basis of calculations applied to data on CO uptake determined by Forbes, Sargent and Roughton (24), Hatch (25) concluded that at equilibrium the partition coefficient of CO between blood and air is of such high magnitude, that the rate of pulmonary blood flow should have a negligible effect on the transfer of gas from alveolar air to blood. These theoretical predictions of Hatch are supported by the results of the present study. A similar lack of relationship between uptake and blood flow would be expected for other gases whose partition coefficients are in the same order of magnitude (26). Consequently, these observations emphasize that as long as permeability of the pulmonary capillary membrane remains high, the factors limiting the uptake of such gases are the size of the diffusing surface and the volume of gas brought to it, rather than the rate of pulmonary blood flow.

With respect to the D_{LCO} as a measure of the size of the capillary bed, it is of interest that a doubling of pulmonary blood flow did not appreciably alter D_{LCO}. Several possibilities may account for a lack of increase in capillary area: *1*) that the capillaries were already distended by the supine position to a point where an increase in blood flow could be accommodated with no further increase in luminal size; *2*) that the increased pulmonary blood flow was accommodated by opening of new capillaries which are in contact with poorly ventilated alveoli; or *3*) that the change in D_{LCO} was too small to be detected by these methods. The data do not allow distinction among these possibilities.

It is easier to rationalize a reduction in $\dot{V}_{CO}$ and D_{LCO} when blood flow is severely curtailed to the point of decreasing perfusion pressures and capillary blood volume. This situation exists distal to an occlusive balloon in a pulmonary artery (27) and such reduction in $\dot{V}_{CO}$ and D_{LCO} have been observed. The possibility arises that stagnation of pulmonary blood distal to the balloon may contribute to the reduction in $\dot{V}_{CO}$ and D_{LCO}. That stagnation is of little significance in this regard is suggested by: *1*) the reduction in D_{LCO} during partial occlusion of a pulmonary artery when flow continues at a considerable, though reduced rate and *2*) the insufficient saturation of pulmonary capillary blood with CO during brief complete occlusion of a pulmonary artery. Thus, it can be shown that in a lung with an assumed capillary blood volume of 30 ml. and a measured CO uptake of 0.4 ml. per minute, the critical saturation of approximately 30 per cent would not be reached during the four minutes of CO breathing.

Relationship between D_{LCO} and pulmonary artery pressure

Pulmonary hypertension from mitral stenosis is associated with an elevation of pressure in the pulmonary capillaries (28). As a consequence, the capillaries may be distended and the area available for diffusion increased. It was observed in this study, that subjects with mitral stenosis had a normal diffusing capacity for CO. This normal value may therefore represent a balance between anatomic alteration of the smaller pulmonary vessels and an increase in the pulmonary capillary blood volume.

In contrast to the pulmonary capillary hypertension of mitral stenosis, the experiments involving unilateral occlusion of a pulmonary artery resulted in a lowering of pulmonary vascular pressure and possibly capillary blood volume distal to the occluding balloon. In these experiments, as would be anticipated, the D_{LCO} of the affected lung was decreased.

Pulmonary blood volume

In the absence of any direct measurement of pulmonary capillary blood volume, changes in this variable can only be inferred. There is indirect evidence (29) that the central blood volume increases in the supine position; the pulmonary capillaries may well share in this increase (30). Such a mechanism was invoked by Bates and Pearce (31) to explain the higher resting diffusing capacity in the supine position for both single breath and steady state methods. The supine position has also been shown to effect a more uniform distribution of blood and inspired gas

in the lungs, particularly with regard to the upper lobes (32).

These considerations suggest that the increase in D_{LCO} during voluntary hyperpnea when blood flow remains virtually unchanged may reflect, in part, an increase in alveolar capillary blood volume. They also indicate that even though pulmonary blood flow has little effect on D_{LCO}, the effect of the volume and distribution of blood in the lung may be appreciable.

The increase in D_{LCO} during exercise

Even though an attempt has been made in this study to isolate some of the individual factors which determine the diffusing capacity of the lung, it is apparent that under most physiological circumstances their interplay is so complicated as to make this type of distinction extremely difficult. This is particularly true of studies done during exercise where ventilation, pulmonary blood flow and possibly pulmonary blood volume all are increased. However, the data from this study do indicate that the increase in diffusing capacity for carbon monoxide, observed during mild to moderate exercise, is related to the increase in ventilation rather than to the increase in pulmonary blood flow. This conclusion is supported by the recent observations of others (33).

SUMMARY AND CONCLUSIONS

1. The effect of minute ventilation and pulmonary blood flow on the diffusing capacity of the lung for carbon monoxide was investigated at rest and during exercise by steady state methods.

2. For this purpose, normal subjects were contrasted with patients in whom pulmonary blood flow had been restricted by mitral stenosis.

3. In order to vary ventilation and blood flow independently, special methods, such as voluntary hyperpnea and unilateral occlusion of a pulmonary artery, were also employed.

4. The results indicate that diffusing capacity for carbon monoxide is little affected by changes in pulmonary blood flow until flow is markedly reduced. By way of contrast, increases in ventilation are associated with increases in diffusing capacity, and seem to account for the rise in diffusing capacity observed during moderate exercise.

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LEFT VENTRICULAR FUNCTION AT REST AND DURING EXERCISE*

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The principle that the degree of ventricular filling at the end of diastole is a determinant of the work done during the succeeding contraction (in the isolated frog heart) stems from Frank (1). Further clarification was achieved by Rohde (2) who noted that ". . . the warm blooded heart is constructed in so complex a manner . . . that it seems hopeless to search for relations between heart volume and length of single muscle fibers. . . . We must [therefore] rearrange the Frank formula . . . so that we can use volume instead of fiber length." Straub (3) was no less explicit when he wrote: "As a specific indication of the length of the muscle elements the volume curve is used as suggested by Frank, and for the representation of tension the pressure curve is used."

Patterson, Piper and Starling (4), acknowledging all the above contributions, concluded that "the increased energy of the heart's contraction under conditions of increased pressure or increased inflow must be ascribed to alterations in the length of the muscle fibres at the beginning of and during their state of excitation, and not to changes in the tension on the fibres." They supported their conclusions relative to isolated canine and tortoise hearts by using a cardiometer for measuring change in ventricular volume and, in effect, used volume as an index to fiber length. The British workers were, therefore, basically in agreement with their German predecessors and colleagues. All seem to have agreed, in addition, that ventricular diastolic pressure was an unreliable guide to ventricular diastolic volume and that the latter, not the former, should be used in the study of intrinsic myocardial responses to stress.

The lack of a method for following changes in ventricular volume in the intact animal or in man made it impossible to test satisfactorily the general applicability of the Frank-Starling principle. Starling, however, thought it might explain the response of the mammalian heart to physical exercise. In his Linacre Lecture (5) he says: "As a result [of exercise] the heart is over filled during diastole, and is impeded from emptying itself in systole: Its volume both in systole and diastole enlarges progressively until by the lengthening of the muscle fibres so much more active surfaces are brought into play within the fibres that the energy of the contraction becomes sufficient to drive on into the aorta during each systole the largely increased volume of blood entering the heart from the veins during the diastole." This logical extension to intact animals of a physiological fact established only for isolated heart preparations was, however, dubious even at the time it was written. Orthodiagraphic and other radiographic techniques, applied to human subjects at rest and during exercise, had in some instances already indicated that total cardiac volume probably decreased as a result of exercise (6–10). Also of more than passing interest in connection with the Frank-Starling principle was von Anrep's finding (11) that adrenalin causes the canine heart to contract more forcibly with no prior increase, or even a decrease, in end-diastolic volume. Such results suggested that the Frank-Starling concept might require modification, if not outright rejection, insofar as the intact mammal is concerned. von Anrep's study certainly raised the possibility that the following concept, as defined by Patterson, Piper and Starling (4), might be an oversimplification: "The output of the heart is a function of its filling, the energy of its contraction depends on the state of dilatation of the heart's cavities."

Subsequently Nylin (12), using more refined roentgenologic methods than had previously been available, showed that cardiac size is maximal when the normal human subject is at rest and

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recumbent; changing from the recumbent to the standing position caused marked reduction in cardiac size. Still later, Liljestrand, Lysholm and Nylin (13) showed that at no time during exercise at increasing loads on a bicycle ergometer did heart size reach the maximum found by Nylin in comparable normal recumbent subjects. They did note, however, that as workloads increased so did cardiac size; but the augmentation of cardiac volume was never as much as would be expected in view of the associated increase in cardiac output per minute. These findings have recently been marshalled by Hamilton (14) as evidence against the general applicability of the Frank-Starling principle.

To this point, all available studies on intact human subjects utilized roentgenologic methods of questionable validity to determine *total* cardiac volume. None attempted or claimed to determine ventricular volume. The development by Rushmer of techniques for using certain left ventricular diameters in intact, unanesthetized dogs as indexes to volume change (15) permitted a more precise scrutiny of left ventricular response to exercise. He found that exercise usually caused a decrease in both systolic and diastolic left ventricular diameters and was of the opinion that the phenomenon was independent of rate changes within the physiologic range.

It has not been possible, to the present time, to follow change in the internal volume of the left ventricle at rest or during exercise in the intact animal or human being. For this reason, a biplane version of a uniplane cinefluorographic technique employed earlier by Rushmer and Thal (16) was adapted for estimation of left ventricular volume. The following study was designed for the purpose of examining the relation between left ventricular end-diastolic volume and stroke work of the succeeding contractions in the intact dog at rest and during exercise.

METHOD

A. Determination of left ventricular volume. The details of the method have been published elsewhere (17). In brief, a biplane cinefluorographic unit of the type devised by Ramsey and co-workers (18) was used to record left and right anterior oblique images of the canine heart at 30 frames per second during the transcardiac passage of radiopaque material. Ventricular images were then projected, using a still projector and the original 35 mm. films, so that the sizes of the images corresponded exactly to that of the actual chamber. Tracings of each pair of images were made, questionable boundaries and valve planes being checked by constant reference to 16 mm. reduction prints of the original 35 mm. films as viewed with a special motion analysis projector. Two methods of calculation were used: One involved inserting the products of the planimetrically determined areas of corresponding left ventricular images into a regression equation developed from studies of casts and models of left ventricular cavities[1]; the other required measurement of corresponding diameters of the two ventricular images at 1 mm. intervals from top to bottom. On the assumption that the left ventricular cavity is elliptical or circular in cross-section, the diameters were then used to calculate the volume of each 1 mm. section of the ventricular cavity:

$$V = \pi h \left(\frac{D_1}{2} \times \frac{D_2}{2} \right),$$

where

V = volume in ml.,
h = 1.0 mm.,
D_1 = ventricular diameter from left anterior oblique view and
D_2 = corresponding diameter from right anterior oblique view.

Total ventricular volume was taken as the sum of the sectional volumes. The volume calculations in the present study were done by use of area products and the regression equation.

Since no independent means of measuring ventricular volume in the living intact animal has been devised, resort was made to elaborate model experiments for validation of the technique. These tests are also presented in detail in the previous description (17). An average error of less than 10 per cent seems likely although the tests, in some respects, are overly severe. Other tests show that under average conditions the technique always stops motion up to 80 cm. per sec.; under optimal conditions, blurring is not encountered until a speed of 110 cm. per sec. is reached.

B. Material and preparation. Six healthy mongrel dogs were employed. They were anesthetized with a mixture of chlorolose (0.05 Gm. per Kg.) and urethane (0.5 Gm. per Kg.) and tracheal cannulation or intubation was carried out as rapidly as possible. A No. 9 cardiac catheter with birdseye tip was then passed into the inferior vena cava just proximal to its junction with the atrium, or into the main pulmonary artery. No. 7 cardiac catheters were then inserted into the femoral or carotid artery and passed retrograde into the left ventricle or aorta (just distal to the aortic valve). In some instances pressures were recorded from both sites through a double lumen catheter. The natural frequency of the system employing a No. 7 catheter (50 cm. long) and a P23D transducer (Statham) is about 12 cps. A slight extension of usable range is obtained by use of mechanical damping. The best system available to us in the present work gives a virtually flat response to 20 cps. It was used to record left ventricular pressures in a few instances.

[1] $\log y = -0.3256 + 0.8062 \log x$, where x = product of 2 ventricular areas and y = ventricular volume.

C. Procedure. The prepared dog was suspended horizontally, feet down, in front of the two fluoroscopic screens, using a special canvas body sling, so that both anterior oblique views of the heart could be recorded. The tracheal tube or cannula was then connected to a Douglas bag via a two-way valve. Arterial and ventricular catheters were connected to transducers which, in turn, were fed into a multichannel photographic electronic recorder. Exercise was administered with a galvanic stimulator the electrodes of which were attached to the upper portions of the hind limbs and to the pelvic region.

The injection catheter was then attached to a pneumatic injector loaded with 90 per cent Hypaque® solution (1.5 ml. per Kg.). Injection of the radiopaque medium was timed, with relation to filming, so that optimal visualization of the left ventricle could be achieved.

Resting runs were done 10 to 15 minutes after the dog was suspended in position. Mild rhythmic exercise was then administered for about 10 minutes. The amount of exercise was such as to cause resting oxygen consumption to increase three to four times. In order to eliminate the effects of body motion on the cinefluorograms, exercise was usually stopped at the instant the cameras were activated. In no instance did filming begin more than two seconds after cessation of exercise.

The two cameras usually are perfectly synchronized. Under some circumstances, however, one camera leads the other by one-sixtieth of a second. When this happens, a warning circuit is activated so that the leading camera can be identified. Corrections are then introduced so that the ventricular images are made synchronous. The photographic pressure tracings bear markings indicating the precise instant at which one of the cameras records its image. In this way, the pressures corresponding to each ventricular volume figure can be identified.

D. Calculations. Ventricular volume and pressure curves are first plotted together (Figures 1 and 2, Tables I and II). Ejection curves are then plotted separately and, if necessary, smooth curves are drawn, in lieu of directly connecting the actual points (Figures 3 and 4). From these, the volume in ml. ejected for each one-thirtieth of a second (ΔV in Tables I and II) can be read off. Aortic cross-sectional area is calculated for each ejection increment by measuring aortic diameters 2 cm. above the aortic valve and applying the equation for the area of a circle or an ellipse. Adjacent values for aortic cross-sectional area are averaged and mean velocity for the interval between each two points on the ejection curve is then calculated. Plots of ejection velocity are thus made possible (Figures 3 and 4). A somewhat similar method utilizing an aortic distensibility curve (and thus taking change in aortic cross-sectional area with pressure into account) was used to construct aortic velocity curves by Remington and Hamilton (19).

Pressure work (W_p)[2] *for each stroke* is first calculated from the conventional work equation:

$$W_p = PV,$$

where

P = mean ejection pressure in Gm. per cm.[2] and
V = total stroke volume in ml.

By plotting left ventricular pressure at each one-thirtieth

[2] The terms *pressure work*, *potential energy* and *pressure-volume work* are by common usage synonymous insofar as the work of the heart is concerned.

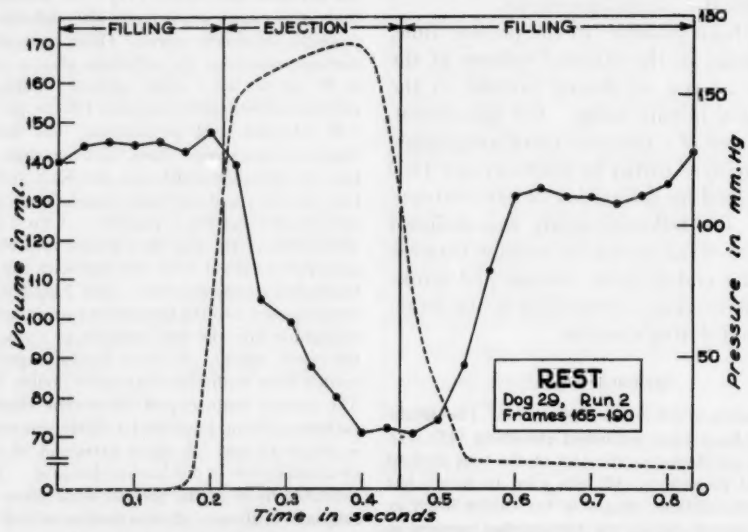

FIG. 1. PRESSURE-VOLUME CURVES THROUGH A CARDIAC CYCLE IN THE ANESTHETIZED RESTING DOG

The solid line refers to volume; the broken line to pressure (see Table I).

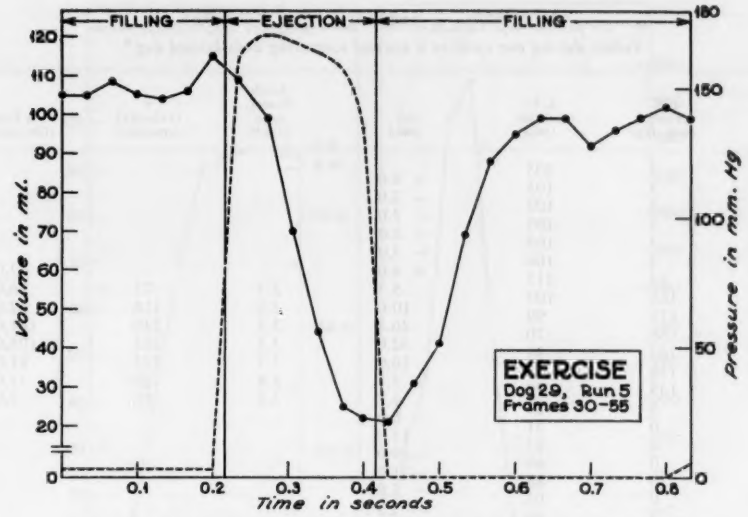

FIG. 2. PRESSURE-VOLUME CURVES THROUGH A CARDIAC CYCLE IN THE ANESTHETIZED EXERCISING DOG

The solid line refers to volume; the broken line to pressure (see Table II).

of a second interval against the corresponding left ventricular volume, one obtains a pressure-volume work loop (Figure 5). The area under the ejection portion of the loop (connected to the zero pressure line by dropping perpendiculars) corresponds to integrated pressure work per stroke:

$$W_p = \int_{t_0}^{t_1} PdV.$$

TABLE I

Values obtained during one cycle in a normal resting anesthetized dog *

Frame number	L.V. pressure (*mm. Hg*)	L.V. volume (*ml.*)	ΔV (*ml.*)	Aortic X-sect. area (*cm.*²)	v (velocity) (*cm./sec.*)	Δ Power (*Gm. cm./sec.*)
165	0	140				
166	0	144	− 1.0			
167	0	145	− 1.0			
168	0	144	− 1.5			
169	0	145	− 1.0			
170	0	141	− 1.0			
171	106	148	− 1.0			−11,000
172	150	139	9.0	2.8	95	48,000
173	157	105	22.5	3.1	218	142,000
174	163	99	17.0	3.2	161	118,000
176	169	80	10.5	3.2	100	72,000
177	169	71	5.8	4.1	42	40,000
178	134	72	2.8	4.1	20	17,000
179	65	71	0.8	3.8	6	3,000
180	39	74	− 4.0			− 8,000
181	11	88	−14.0			−14,000
182	11	112	−23.5			−10,000
183	11	131	−19.3			− 8,000
184	11	133	− 1.7			− 1,000
185	11	131	0			
186	11	131	0.5			
187	8	129	− 0.5			
188	8	131	− 0.5			
189	8	134	− 0.5			
190	8	142	− 1.0			

* These data are the basis on which Figures 1, 3, 4, 5 and 6 are constructed.

TABLE II

Values during one cycle in a normal exercising anesthetized dog *

Frame number	L.V. pressure (*mm. Hg*)	L.V. volume (*ml.*)	ΔV (*ml.*)	Aortic X-sect. area (*cm.²*)	v (velocity) (*cm./sec.*)	Δ Power (*Gm. cm./sec.*)
30	3	105	− 4.0			
31	3	105	− 3.0			
32	3	107	− 2.0			
33	3	105	− 2.0			
34	3	104	− 3.0			
35	3	106	− 4.0			−2,000
36	22	115	5.5	2.3	72	16,000
37	161	109	10.0	2.6	116	52,000
38	171	99	26.5	3.2	249	161,000
39	168	70	32.0	3.5	272	198,000
40	164	44	16.0	3.9	123	81,000
41	158	25	2.5	3.8	20	11,000
42	135	22	1.2	3.6	10	2,000
43	0	21	− 6.0			
44	0	31	−13.0			
45	0	41	−29.0			
46	0	69	−21.0			
47	0	88	− 3.0			
48	0	95	− 1.5			
49	0	99	− 1.5			
50	0	99	− 1.0			
51	0	92	− 1.0			
52	0	96	− 1.0			
53	0	99	− 1.0			
54	0	102	− 1.0			
55	6	99				

* These data are plotted in Figures 2, 3, 4, 5 and 6.

In actual practice, volume in ml. is plotted on the abscissa and pressure (in mm. Hg) is plotted on the ordinate. The area under the ejection portion of the loop is measured planimetrically and correcting factors are applied to reduce the area to fundamental terms. The factor 1.355 is introduced in order to convert mm. Hg to Gm. per cm.². The

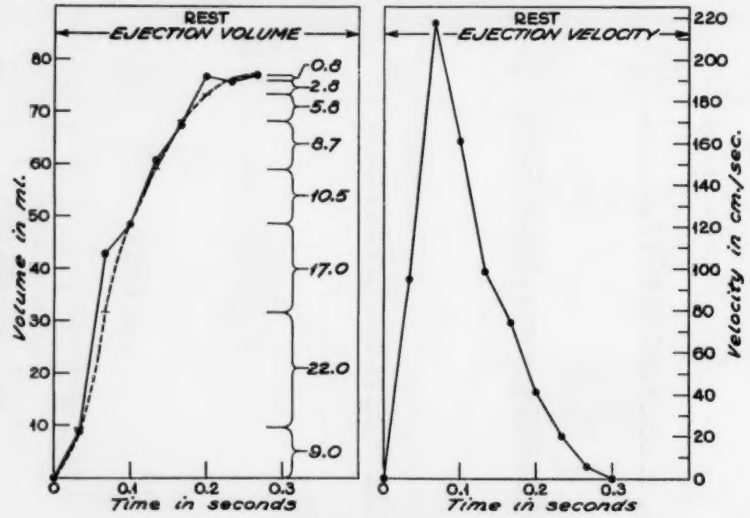

FIG. 3. RESTING EJECTION CURVE (LEFT) AND VELOCITY CURVE (RIGHT)

The observed ejection curve is represented by the solid line. The broken line represents the curve, fitted free hand to the available data, from which velocity was calculated (see Table I).

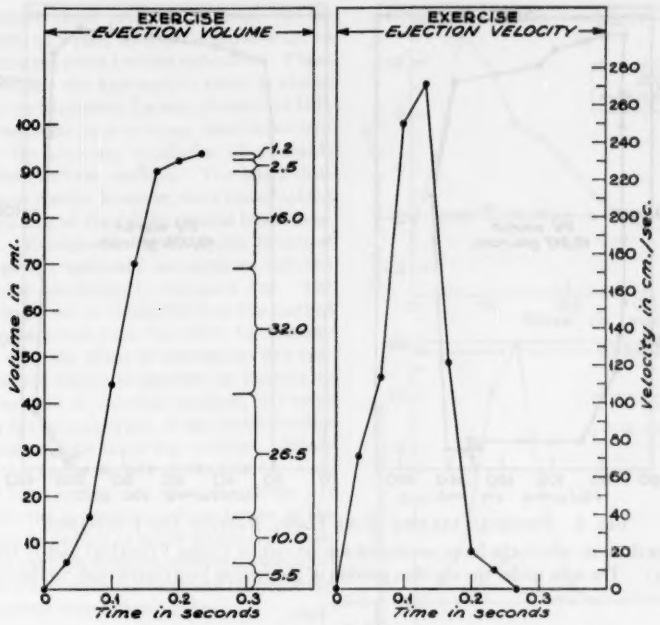

FIG. 4. EXERCISING EJECTION (LEFT) AND VELOCITY (RIGHT) CURVES
See Table II.

final value is in Gm. cm. units (Gm. per cm.^{2} × cm.^{3} = Gm. cm.). For example, W_p was calculated from the resting loop in Figure 5 by plotting volume on the abscissa so that 10 ml. = 1.0 cm. paper, and pressure on the ordinate so that 10 mm. = 1.0 cm. paper. The area under the ejection portion of the loop was 119.68 cm.^{2}. This, multiplied by 135.5 (1.355 × 100), gives 16,217 Gm. cm. The data on which the loop is based are given in Table I.

Kinetic work (W_k)[3] *per stroke* may also be calculated from mean values:

$$W_k = \frac{mv^2}{2g},$$

where

m = total stroke mass in Gm. (total stroke volume in ml. × 1.06),
v = mean stroke velocity in cm. per sec. and
g = 980 cm. per sec.^{2}.

In order to obtain the value

$$W_k = \int_{t_0}^{t_1} \frac{v^2 dm}{2g},$$

v^2 for each one-thirtieth of a second interval must be plotted against mass ejected during the same interval. The area under the resulting curve, when appropriately converted to absolute units, yields integrated kinetic work in Gm. cm. For example, W_k for the cycle shown in Figure 6 is obtained by plotting v^2 (Table I) against mass ejected for each one-thirtieth of a second during ejection. Mass, on the abscissa, is plotted so that 10 Gm. = 1 cm. Velocity squared, on the ordinate, is plotted so that 5,000 cm.^{2} per sec.^{2} = 1 cm. The area under the curve is 34.0 cm.^{2}. This value is multiplied by 5,000 × 10/1,960, or 25.51, to yield W_k in Gm. cm. In this case, the answer is 867.3 Gm. cm.

Total stroke work is, of course, the sum of W_p and W_k, whether calculated from mean or integrated values.

A power curve representing the rate at which work is done during the cardiac cycle is obtained if total work ($W_p + W_k$) done on blood in each one-thirtieth of a second interval during ejection is plotted against elapsed time from the beginning of ejection. The course of power development and the relation between pressure and kinetic power at rest and during exercise are shown in Figure 7. Power may also be obtained as the first derivative of the *cumulative* work curve. The fundamental difference between work and power is made apparent by comparing Figures 6 (work) and 7 (power).

E. Possible sources of error. The error inherent in the method itself, as defined previously (17), is small enough to be acceptable for present purposes. The most serious remaining objection to the technique is the cardiovascular effect of highly concentrated contrast medium, delivered very rapidly into the vena cava or pulmonary artery. It has been shown by Muirhead, Lackey, Bunde and Hill (20) that rapid injection of highly concentrated solutions into the heart often produces bradycardia, an effect which

[3] Also known as *kinetic energy, acceleration work* or *energy of velocity.*

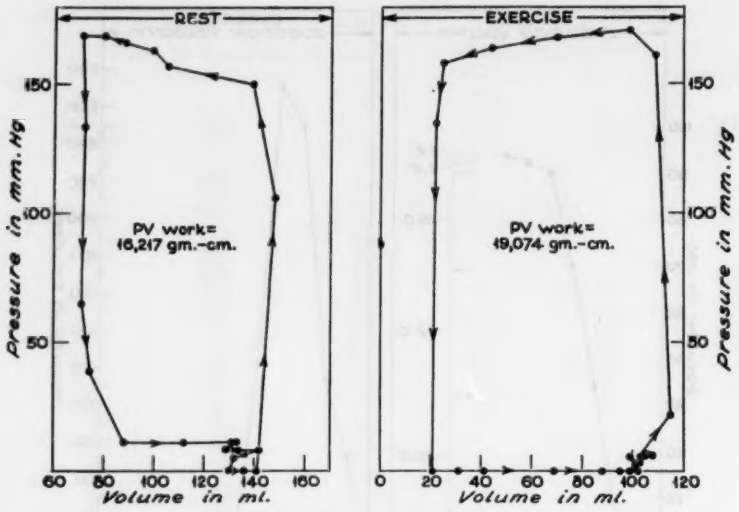

FIG. 5. PRESSURE-VOLUME WORK LOOPS, RESTING AND EXERCISING

The data on which the loops are based are set out in Tables I (resting) and II (exercising). The area under the ejection portion of the resting loop corresponds to the value

$$\int_{V=75}^{V=145} PdV;$$

on the right, the corresponding value is

$$\int_{V=20}^{V=110} PdV.$$

is abolished by vagotomy, as shown by Deyrup and Walcott (21). Some degree of arterial hypotension has also been observed following such injections. Both effects were frequently seen in the present studies. Bradycardia occurred most frequently when injection was into the pulmonary artery and sometimes abolished the usual increase in pulse rate produced by exercise. It occurred almost immediately after contrast medium reached the pulmonary

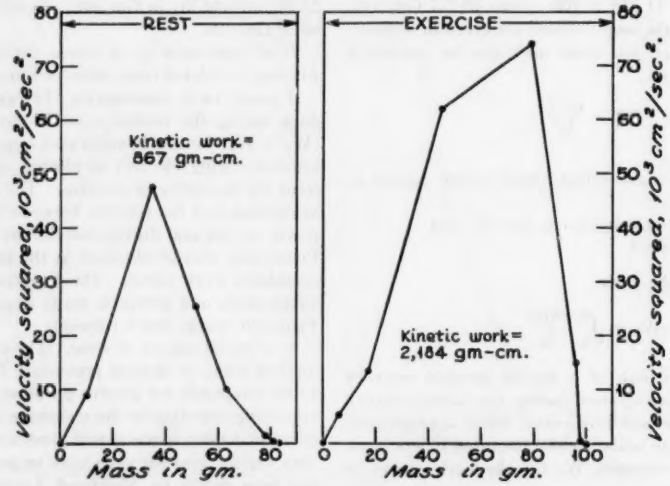

FIG. 6. KINETIC WORK CURVES AT REST AND DURING EXERCISE

See Tables I and II for data used to construct the curves.

artery. Fall in arterial blood pressure, however, was invariably delayed and, as a rule, several measurable cycles had taken place before the effect became noticeable. There is reason to believe that the hypotensive effect is attributable, as suggested by Muirhead, Lackey, Bunde and Hill, to decline in left ventricular stroke volume, itself dependent on the arrival at the coronary capillaries of a sizable concentration of the contrast medium. The effect does not vitiate the present results, however, since cycles taking place after the beginning of the fall in arterial blood pressure were deleted. Enough unaffected cycles remained (three to 10) to permit adequate comparisons between resting and exercising conditions in the same dog. The bradycardic effect could not be eliminated but was marked in only two dogs. In actual fact, the effect fortuitously provided evidence that the effect of exercise on left ventricular function is not wholly dependent on increase in pulse rate. The volume of contrast medium, delivered relatively suddenly into some portion of the central cardiovascular system, must also be taken into account. Whatever the effect of this factor on left ventricular function, it was common to both resting and exercising states. It is also probable that the increase in volume return to the left ventricle was not as sudden as might be surmised from the total volumes of contrast medium injected; since injection required 1.5 to 2.0 seconds, the increased return to the left ventricle was spread over several cycles.

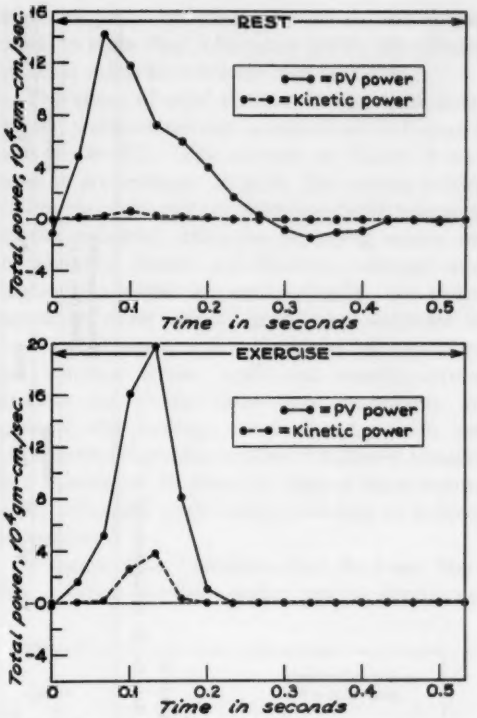

FIG. 7. POWER CURVES AT REST AND DURING EXERCISE

RESULTS

Table III shows pulse rates and mean values for left ventricular end-diastolic, stroke and minute volumes at rest and during exercise. The pulse rates are those that obtained as the actual cycles that were used for volume calculations took place.

Stroke work and minute work, calculated by both methods (using mean values, on the one hand, and the integration procedure, on the other), are shown in Table IV. The pulse rates given in Table III were used to convert stroke to minute work.

A sample plot of total stroke work at rest against end-diastolic volume is set out in Figure 8. A similar plot, showing both resting and exercising values in a normal dog, without left ventricular failure, is seen in Figure 9. Identical

TABLE III

Left ventricular end-diastolic, stroke and minute volume for entire normal group *

Dog number	Wt. (*Kg.*)	End—diastolic volume (*ml.*) Rest	Exercise	Stroke volume (*ml.*) Rest	Exercise	Pulse rate Rest	Exercise	Minute volume (*L./min.*) Rest	Exercise
13	15.8	80 ± 2.8	68 ± 5.0	28 ± 2.7	34 ± 6.6	125	160	3.2 ± 0.3	5.1 ± 0.9
14	17.3	66 ± 9.1	56 ± 14.8	29 ± 10.8	28 ± 1.8	136	210	3.9 ± 1.4	5.6 ± 0.3
15	17.3	83 ± 5.4	71 ± 6.5	27 ± 4.6	33 ± 7.2	160	160	4.0 ± 0.7	5.1 ± 1.1
26	22.3	126 ± 14.0	107 ± 6.8	77 ± 13.0	73 ± 8.3	90	84	6.9 ± 1.6	5.4 ± 1.0
29	24.6	138 ± 5.2	107 ± 4.4	84 ± 4.7	83 ± 5.4	66	82	5.0 ± 1.2	6.2 ± 0.9
30	20.2	126 ± 4.2	96 ± 6.2	71 ± 9.7	72 ± 7.8	55	99	4.2 ± 1.3	6.9 ± 0.9
Mean		100 ± 28.2	89 ± 19.9	49 ± 26.4	59 ± 22.4			4.6 ± 1.7	5.9 ± 1.2
n		37	32	37	32			37	32
σ_D		5.8		5.9				0.35	

* The n refers to total cycles used to derive the mean values.

TABLE IV

Left ventricular work data for the entire material

Dog number	Integrated stroke work (*1000 Gm. cm.*)						Mean stroke work (*1000 Gm. cm.*)					
	Resting			Exercise			Resting			Exercise		
	PE	KE	Total	PE	KE	Total	PE	KE	Total	PE	KE	Total
13	3.3 ± 0.4	0.1 ± 0.04	3.4 ± 0.4	5.0 ± 0.6	0.3 ± 0.20	5.4 ± 0.8	3.3 ± 0.4	0.07 ± 0.03	3.4 ± 0.4	4.7 ± 0.6	0.2 ± 0.10	4.9 ± 0.7
14	4.8 ± 2.0	0.1 ± 0.08	4.9 ± 2.0	4.4 ± 0.5	0.1 ± 0.05	4.6 ± 0.6	4.8 ± 2.2	0.07 ± 0.06	4.8 ± 2.3	3.3 ± 0.2	0.05 ± 0.010	3.5 ± 0.4
15	5.1 ± 1.1	0.07 ± 0.07	5.1 ± 1.1	6.0 ± 1.5	0.2 ± 0.10	6.2 ± 1.6	4.9 ± 1.0	0.03 ± 0.01	4.9 ± 1.0	5.6 ± 1.2	0.1 ± 0.07	5.7 ± 1.2
26	11.7 ± 2.7	0.7 ± 0.30	12.4 ± 2.9	9.5 ± 1.6	1.8 ± 0.90	11.3 ± 2.4	13.2 ± 2.7	0.4 ± 0.10	13.6 ± 2.9	9.4 ± 1.5	0.7 ± 0.30	10.1 ± 2.0
29	15.2 ± 0.9	0.7 ± 0.30	15.9 ± 1.1	17.5 ± 1.7	1.5 ± 0.40	18.9 ± 1.8	15.2 ± 1.3	0.4 ± 0.08	15.6 ± 0.8	17.2 ± 1.6	0.7 ± 0.20	17.9 ± 1.5
30	9.1 ± 1.2	0.7 ± 0.08	9.8 ± 1.2	13.4 ± 1.8	1.4 ± 0.50	14.8 ± 2.1	9.5 ± 1.7	0.4 ± 0.09	9.9 ± 1.8	13.6 ± 2.2	0.9 ± 0.30	14.5 ± 2.3
Mean	7.8 ± 4.4	0.4 ± 0.40	8.2 ± 4.7	10.4 ± 4.9	1.1 ± 0.80	11.5 ± 5.5	8.1 ± 4.8	0.2 ± 0.20	8.3 ± 4.9	10.1 ± 5.1	0.5 ± 0.40	10.7 ± 5.4
n (cycles)		37			32			37			32	

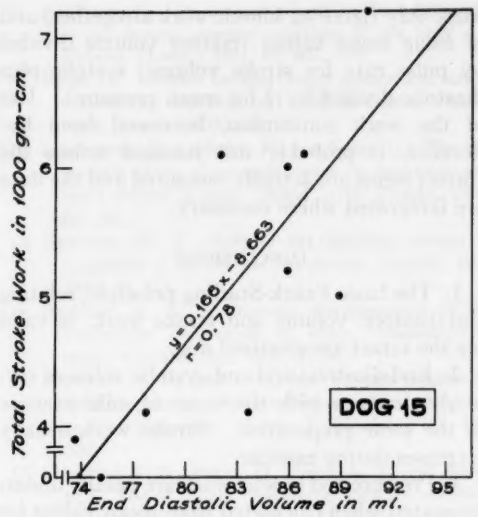

FIG. 8. RELATION BETWEEN LEFT VENTRICULAR END-DIASTOLIC VOLUME AND STROKE WORK DURING 10 CONSECUTIVE CYCLES IN A RESTING, ANESTHETIZED DOG WITH NO EVIDENCE OF LEFT VENTRICULAR FAILURE

qualitative relations prevailed in five other dogs. Attempts to relate left ventricular end-diastolic pressure and stroke work, at rest or during exercise, were unsuccessful. In Dog 15, for example, end-diastolic pressure was zero for 10 successive resting cycles, yet stroke work varied from 3.6 to 7.2 thousand Gm. cm. Owing, however, to the fact that variations in intrapleural pressures were not measured, the figures shown are not *effective* pressures. It is therefore not possible to use the data to establish or deny a direct relation in the intact animal between end-diastolic pressure and work done in the succeeding contraction.

DISCUSSION

The data presented confirm the basic Frank-Starling hypothesis for the intact resting dog: A definite relation exists, between left ventricular *end-diastolic volume* and the work of the stroke immediately following, provided conditions are held reasonably constant while the observation is being made. The plot shown in Figure 8 is representative of the relationship as disclosed by the method in resting, intact, anesthetized dogs. Spontaneously occurring changes in ventricular function under these conditions provide a sufficient range of end-diastolic volume and stroke work to show that a linear or gently curvilinear relation exists between the two variables.

The effect of mild exercise in the nonfailing, intact, anesthetized dog is well shown in Figure 9 and Table III. The pattern in Figure 9 was seen in six animals; in each, the resting values fall to the right and are quite low (with reference to the ordinate), while the exercising values are to the left (lower end-diastolic volume) and higher (in terms of stroke work). In other words, exercise usually produces a decrease in both end-diastolic and end-systolic left ventricular volume; stroke work and usually stroke volume are, at the same time, increased. In general, the findings are in keeping with the older orthodiagraphic studies and closely parallel the results of Rushmer's elegant experiments (15), using left ventricular diameters as indexes to volume.

It seems clear, therefore, that the basic Starling relation prevails under resting conditions

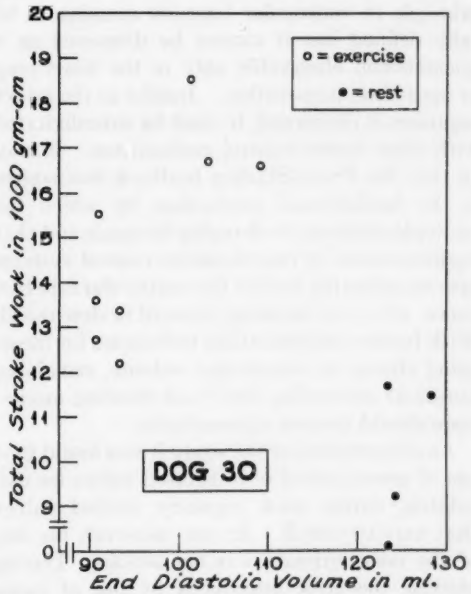

FIG. 9. PLOTS OF RESTING VALUES FOR THE NONFAILING LEFT VENTRICLE (END-DIASTOLIC VOLUME VERSUS STROKE WORK) COMPARED TO A PLOT OF EXERCISING VALUES

Note the decline in end-diastolic volume (abscissa) and increase in stroke work (ordinate) occasioned by exercise.

and that it may also be operative during mild exercise. But the fact that end-diastolic volume during exercise is normally less than at rest, at a time when stroke work is actually increased, indicates that exercise imposes some basic functional difference on the nonfailing ventricle. The data reported may conceivably represent segments of ventricular function curves, the exercise curve lying above and to the left of the resting. If this is true, the results are in keeping with Sarnoff and Berglund's ingenious concept (22, 23) of a "family of curves," each one representing a different set of conditions under which the ventricle is operating. The results do not actually define a curve of any sort but clearly show that the ventricle is operating at different functional levels, as defined by the relation between end-diastolic volume and stroke work, at rest and during exercise. The data fit equally well on the set of curves as drawn by Hamilton (14) but provide no information concerning a descending limb.

The ultimate role of the basic Frank-Starling principle in ventricular function remains to be fully defined but it cannot be dismissed as a phenomenon observable only in the heart-lung or open chest preparation. Insofar as the intact organism is concerned, it must be interdigitated with other known control mechanisms. It may be that the Frank-Starling feedback mechanism is the fundamental mechanism by which the ventricle responds to changing demands and that various neural or neurohumeral control systems operate primarily to shift the ventricular function curve, whatever its shape, upward or downward. With further refinements in techniques for measuring change in ventricular volume, remaining questions concerning the Frank-Starling mechanism should become approachable.

As a by-product of the study it was found that use of mean instead of integrated values for calculating stroke work regularly yielded values that were too small. At rest, however, the size of the underestimate was not serious. During exercise the error introduced by use of mean values becomes more consequential, especially when one considers the kinetic work category (Figure 7). The strong probability is that for any but resting conditions it is hazardous to follow the usual practice of calculating pressure work only (ignoring kinetic work altogether) and of using mean values (minute volume divided by pulse rate for stroke volume; systolic plus diastolic divided by 2 for mean pressure). Use of the work convention, borrowed from hydraulics, is probably not justified unless the correct items are actually measured and the data are integrated where necessary.

CONCLUSIONS

1. The basic Frank-Starling principle, relating end-diastolic volume and stroke work, is valid for the intact anesthetized dog.

2. End-diastolic and end-systolic volumes regularly decrease with the onset of mild exercise in the same preparation. Stroke work usually increases during exercise.

3. Pressure and kinetic work are usually underestimated when calculated from mean values for pressure, volume and velocity instead of from integrated data.

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CHANGES IN RENAL CONCENTRATING ABILITY PRODUCED BY PARATHYROID EXTRACT *

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Polyuria is a frequent symptom of hyperparathyroidism. Impairment of renal concentrating ability, often out of proportion to other signs of renal insufficiency, has been noted in patients with hyperparathyroidism (1–3) as well as in patients and animals with hypercalcemia due to other causes (4–6). In many patients with hyperparathyroidism, extensive deposits of calcium in the renal parenchyma, secondary fibrosis, superimposed pyelonephritis and vascular disease have combined to destroy considerable amounts of renal substance. The mechanism of the reported decrease in renal concentrating ability in such patients is not clear, since fixation of urinary specific gravity is a well-known accompaniment of the simple ablation of renal tissue (7).

In the present experiments, renal concentrating capacity was found to be depressed in dogs in which hypercalcemia had been induced for only 24 hours by injecting parathyroid extract. This functional defect was associated with morphologic changes in the distal portions of nephrons and the collecting ducts.

MATERIALS AND METHODS

Mature female dogs weighing 8 to 22 Kg. were maintained on Purina Chow® supplemented with horsemeat. All experiments were carried out under light pentobarbital anesthesia. Inulin was determined by the method of Walser, Davidson and Orloff (8), osmolality of serum and urine with a Fiske osmometer, serum calcium according to Kingsley and Robnett (9), and sodium in serum and urine by flame photometry.

Group I. Studies of concentrating ability during graded osmotic diuresis

Four dogs were studied. Prior to the experiment, food and water were withheld for 24 hours. Five units of vasopressin in oil [1] was injected subcutaneously on the evening before the experiment. A control study was carried out on each dog one week before it received parathyroid extract. A total of 90 units of parathyroid extract [2] per Kg. was injected subcutaneously in three divided doses during the 24 hours preceding the second study. During the experiment, 0.5 per cent inulin in physiological saline was infused at a rate of 1 to 1.5 ml. per minute, together with 50 to 100 milliunits of vasopressin [1] per Kg. per hour. Samples of urine and blood were collected at 10 to 15 minute intervals. After a suitable period of equilibration, urine flow was slowly increased from less than 1 ml. per minute to more than 10 ml. per minute by gradually increasing the rate of infusion of 5 per cent mannitol in 0.72 per cent saline. This technique permitted examination of the concentrating function over a wide range of urine flows and rates of solute excretion.

Group II. Studies of concentrating ability during a constant infusion of mannitol

Seven dogs were studied before and after receiving parathyroid extract in doses comparable to those given to the preceding group. Five per cent mannitol in 0.72 per cent saline was infused at a rate of 0.50 to 0.75 ml. per Kg. per minute throughout the experiment. Inulin and vasopressin were infused simultaneously as in Group I. Following an equilibration period of one hour, when urine flow had reached a constant value, collections of urine were made every 10 to 15 minutes. Samples of blood were obtained from the jugular vein at intervals of 20 to 30 minutes. Three dogs were restudied in the same way at intervals up to five weeks after treatment with hormone.

In six additional dogs not given parathyroid extract, the effect of progressive osmotic loading on $T^{c}H_{2}O$ was studied by increasing the rate of infusion of 5 per cent

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|| Fellow of the Life Insurance Medical Research Fund.

¶ Advanced Fellow of the American Heart Association.

** Special Fellow of the Rockefeller Foundation.

[1] Supplied through the courtesy of Parke, Davis and Co. and Dr. J. E. Gajewski.

[2] Paroidin® (Parke, Davis and Co.) was kindly supplied by Dr. D. A. McGinty.

mannitol in saline stepwise to three or four times the initial rate of 0.50 to 0.75 ml. per Kg. per minute.

Dogs were autopsied at intervals up to two months after receiving parathyroid extract. The kidneys were fixed in formalin and studied by microdissection as well as by standard histological techniques.

RESULTS

Subcutaneous injection of 60 to 97 units of parathyroid extract per Kg. over a period of 24 hours resulted in *an increase in serum calcium* to 15 to 20 mg. per 100 ml. During this time the animals usually looked sick and listless and exhibited polyuria. Serum calcium returned to normal after 48 to 72 hours and the dogs had generally regained their usual vigor by one week after injections of parathyroid extract were discontinued.

Glomerular filtration rate (*GFR*) usually decreased, but remained essentially unchanged in two dogs and rose in one animal, following the administration of parathyroid extract.

The *maximum urinary concentration rate* (U_{osm}/P_{osm}) achieved before mannitol loading in dehydrated dogs injected with vasopressin in oil was considerably diminished following parthyroid extract (Figure 1) but tended to rise to or toward the control level several days to weeks after the serum calcium had fallen to normal.

Studies of *concentrating ability during graded osmotic diuresis* (Group I) demonstrated impairment of water conservation at all levels of urine flow and solute load. The four experiments of this group are summarized in Figures 2A through 2D. Despite the continuous infusion of large amounts of exogenous vasopressin, urine became hypotonic to plasma at relatively low levels of solute excretion in three out of four experiments and the excretion of free water increased thereafter with increasing solute diuresis.

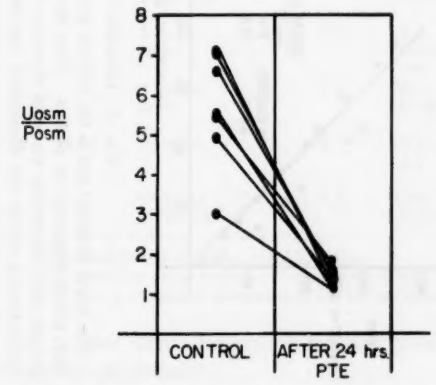

FIG. 1. DECREASE IN MAXIMUM OSMOLAL U/P RATIO AFTER TREATMENT WITH PARATHYROID EXTRACT (PTE)

Similar changes in T^cH_2O were observed in the experiments of Group II (Figure 3, Table I). Hypotonic urine was excreted during moderate mannitol loading in five of seven dogs. Solute loads of much greater magnitude in proportion to GFR did not suffice to produce hypotonic urine in six normal dogs infused rapidly with 5 per cent mannitol in 0.72 per cent saline (Figure 4).[3]

In experiments of Group I and Group II, the ratio of urinary sodium to total urinary solutes was not consistently altered by parathyroid extract.

Lower doses of parathyroid extract (30 to 35 units per Kg.) produced elevations of serum calcium of only 1 to 2 mg. per 100 ml. in two dogs, with no changes in renal concentrating ability or glomerular filtration rate.

The subcutaneous injection of 4.5 to 7.0 Gm. of calcium as calcium acetate into three animals resulted in increased urinary excretion of calcium. Serum calcium levels were slightly elevated two to four hours after the injection but had returned to normal by the following day. At this time T^cH_2O was found to be unaltered.

Morphological changes

Dogs autopsied within a few days after receiving parathyroid extract had focal tubular lesions of irregular patchy distribution. These were demonstrated by microdissection to lie along the *ascending limbs of Henle's loop*, the *distal convolutions* and the *collecting system*. They consisted of foci of hydropic swelling of the tubular epithelial cells, acute necrosis of the epithelium, frequently with calcification, and often calcification of basement membranes. The necrotic, calci-

[3] Others (10, 11) have reported that T^cH_2O may disappear during massive osmotic diuresis in normal dogs. We have observed hypotonic urines in normal dogs loaded with mannitol in saline only with solute loads in excess of those depicted in Figure 4. A possible explanation for the maintenance of hypertonic urine during osmotic diuresis in our normal dogs may lie in their high protein diet (12).

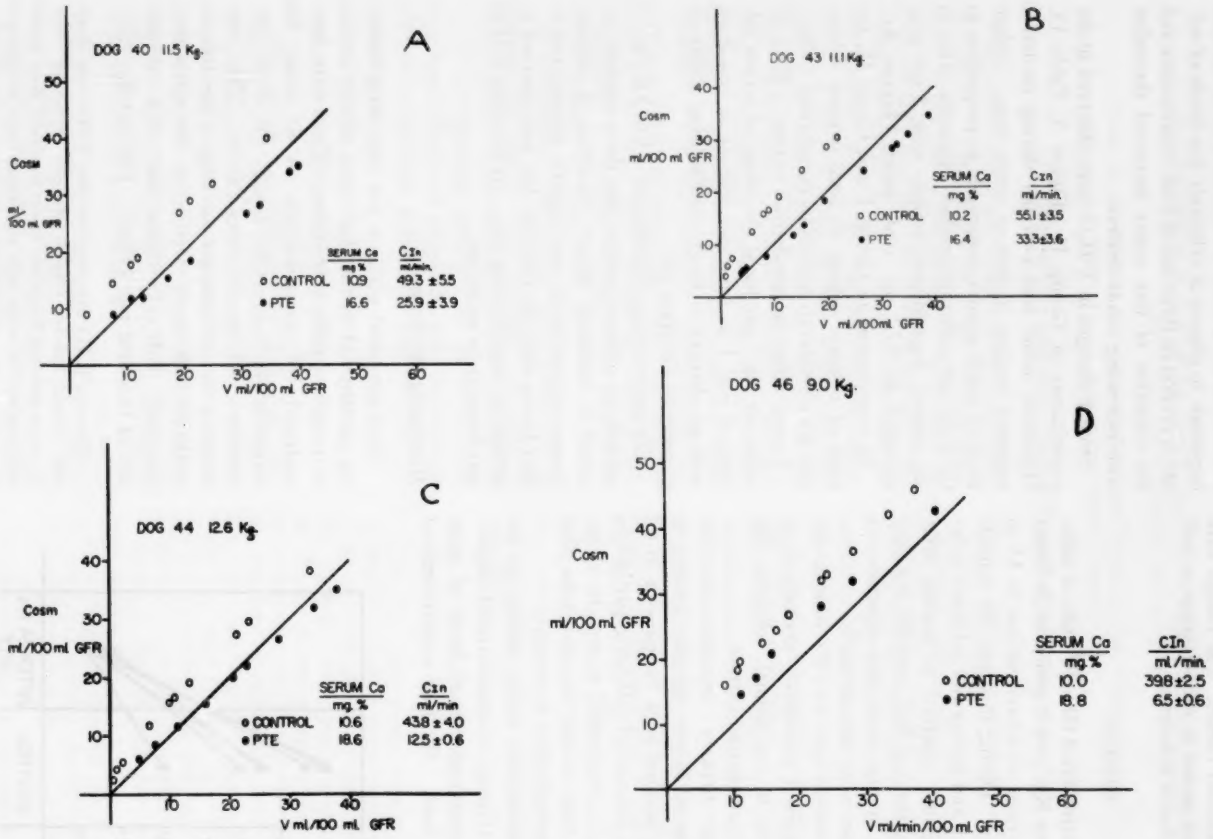

FIG. 2. IMPAIRMENT OF T^cH_2O AFTER TREATMENT WITH PARATHYROID EXTRACT (PTE)

The heavy diagonal line is the isotonic parameter. Points above this line represent urines more concentrated than plasma; points below the line, urines hypotonic to plasma. Note that after administration of parathyroid extract (black dots), urine became progressively more hypotonic with increasing mannitol diuresis, despite a constant intravenous infusion of vasopressin. In D note that after parathyroid extract, concentrating ability was diminished, but urine hypotonic to plasma was not produced even during heavy osmotic diuresis. It is noteworthy that this dog had the most severe impairment in glomerular filtration of all four animals in Group I.

TABLE I

Effects of parathyroid extract on renal function (Group II)

Dog No.	Wt.	Procedure	No. of periods	Serum Ca*	V	Uosm V	T^cH_2O†	C_{In}	$\frac{U_{Na}}{Uosm}$	$\frac{T^cH_2O†}{C_{In}} \times 100$	Maximum $\frac{Uosm}{Posm}$
	Kg.			*mg./100 ml.*	*ml./min.*	*mOsm./min.*	*ml./min.*	*ml./min.*			
3	8.4	Control	4	9.7	7.5‡ ±0.5	2,575 ±56	0.5 ±0.17	29 ±2.0	0.28	1.7 ±0.83	
		90 units PTE/Kg.	5	13.1	11.0 ±0.5	3,474 ±236	−1.1 ±.01	27 ±1.2	0.31	−4.2 ±0.39	
		9 days later	5	8.4	10.7 ±1.2	3,878 ±387	1.0 ±0.30	28 ±0.8	0.30	3.6 ±0.40	
4	18.6	Control	5	8.7	11.9 ±1.6	5,118 ±607	4.3 ±0.48	65 ±7.2	0.25	6.6 ±0.15	
		77 units PTE/Kg.	4	14.7	16.9 ±0.94	6,449 ±379	2.7 ±0.14	83 ±3.3	0.25	3.3 ±0.14	
5	20.7	Control	5	10.5	19.0 ±1.0	7,407 ±265	4.5 ±0.21	80 ±3.2	0.32	5.7 ±0.41	
		97 units PTE/Kg.	5	15.0	6.7 ±0.48	2,039 ±188	−0.4 ±0.08	13 ±0.51	0.29	−3.4 ±0.61	
8	13.5	Control	5	10.4	9.0 ±0.68	3,905 ±243	3.6 ±0.25	70 ±8.3	0.25	5.2 ±0.38	4.9
		60 units PTE/Kg.	5	16.0	11.7 ±1.0	3,760 ±357	−0.4 ±0.08	34 ±1.2	0.27	−1.1 ±0.19	1.1
9	17.5	Control	5	8.9	17.7 ±0.96	7,134 ±339	5.0 ±0.19	73 ±3.7	0.28	6.9 ±0.11	5.4
		60 units PTE/Kg.	5	12.3	20.1 ±1.2	6,619 ±150	−0.5 ±0.14	53 ±1.5	0.32	−1.0 ±0.27	1.8
		5 weeks later	6	7.6	15.6 ±1.7	6,427 ±623	4.4 ±0.22	70 ±2.1	0.26	6.3 ±0.38	5.7
19	15.0	Control	4	9.4	11.0 ±0.29	4,736 ±25	3.9 ±0.17	49 ±1.4	0.31	7.9 ±0.43	3.0
		60 units PTE/Kg.	5	14.5	4.2 ±0.20	1,463 ±105	0.2 ±0.10	10 ±0.42	0.28	2.4 ±0.98	1.1
		8 days later	4	7.0	5.8 ±0.30	2,011 ±99	0.1 ±.08	13 ±0.24	0.22	0.6 ±0.59	1.8
		23 days later	4	7.6	10.4 ±0.36	3,633 ±109	0.4 ±0.15	26 ±0.42	0.26	1.4 ±0.58	2.5
6	16.0	Control	7	8.7	10.5 ±2.5	4,374 ±834	3.2 ±0.21	49 ±3.2	0.26	6.6 ±0.58	
		94 units PTE/Kg.	7	13.0	12.4 ±0.82	3,873 ±313	−0.5 ±0.15	22 ±1.1	0.32	−2.3 ±0.68	

* During infusion of mannitol in saline.
† A negative value indicates urine more dilute than plasma.
‡ Mean ± standard deviation of all the periods listed in Column 4.

fied, cellular debris formed obstructing casts with dilatation proximally.

In the kidneys of dogs autopsied two weeks to two months after receiving parathyroid extract there was "fatty change" of the tubular epithelium similar in distribution to the early alterations. Proliferative lesions, seen only in the medullary portion of the collecting system, consisted of intratubular cellular masses, often calcified, which produced obstruction and proximal dilatation, and

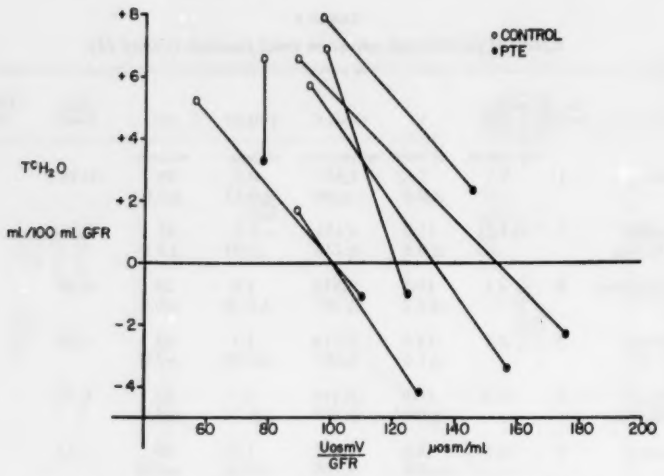

FIG. 3. EFFECT OF PARATHYROID EXTRACT (PTE) ON T^cH_2O

After 24 hours of treatment with parathyroid extract, T^cH_2O was greatly diminished in all animals; in five, hypotonic urine was elaborated despite the administration of vasopressin.

in places extended beyond the tubular basement membrane into the connective tissue stroma.

Chemical analysis of the kidneys in two instances (Table II) demonstrated an increased calcium content of the renal medulla. The amount of calcium in the renal cortex was not greatly altered by injections of parathyroid extract.

These changes will be described more fully elsewhere (13). The character and distribution of the renal lesions resembled those observed in rats intoxicated with vitamin D (6).

TABLE II

Calcium content of serum, urine and kidneys of dogs treated with parathyroid extract and of normal dogs *

	Dog No.	Serum Ca	Urine Ca	Ca content, mg./Kg. FFWT†	
				Cortex	Medulla
		mg. %	*mg. %*		
Parathyroid	8‡	16	6	179	832
	19‖	7	7	173	1,007
Normal	24	6	2	188	141
	25	7	4	116	103

* All animals were sacrificed while undergoing mannitol diuresis.

† Fat-free wet tissue.

‡ Sacrificed at the conclusion of the experiment summarized in Table I after 24 hours of parathyroid extract.

‖ Twenty-three days after parathyroid extract.

DISCUSSION

Renal function is often impaired in hyperparathyroidism (14) and although renal insufficiency may prove reversible (3, 14, 15) it frequently progresses even after the concentration of calcium in the serum is restored to normal by parathyroidectomy. Edvall (14) found the clearances of inulin and para-aminohippurate (PAH) as well as T_mPAH to be depressed in most but not all patients with hyperparathyroidism. Hellström (2) noted that patients with this disease frequently excreted urine with maximum specific gravity below normal, even when azotemia was absent. After operation, the ability to concentrate the urine was often restored. Cohen, Fitzgerald, Fourman, Griffiths and deWardener recorded maximum urinary concentrations below the concentration of solutes in plasma in two patients with hyperparathyroidism who in addition had reduced levels of glomerular filtration rate and (in one) a decreased renal plasma flow (3).

The present experiments suggest that the ability of the kidneys to produce a concentrated urine was impaired after only 24 hours of hypercalcemia produced by injections of parathyroid extract. Interpretation of the data is complicated by the fact that in most cases glomerular filtration rate,

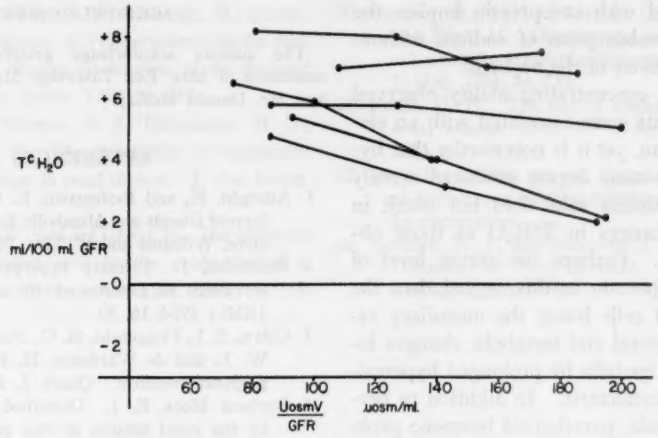

FIG. 4. EFFECT OF PROGRESSIVE MANNITOL LOADING ON NORMAL DOGS

Massive solute diuresis was associated with some decline in T^cH_2O but did not, in these experiments, result in the appearance of urine hypotonic to plasma.

was also reduced by parathyroid extract. *Acute* reductions in glomerular filtration rate, induced by compressing the renal artery of dogs undergoing mannitol diuresis, result in a rise, rather than a fall, in maximum urinary osmolality (16). Nonspecific destruction of renal tissue, whether produced by injecting an aminonucleoside into the renal artery, by experimental pyelonephritis or by temporary renal ischemia, does not appreciably reduce the ratio of T^cH_2O to glomerular filtration rate, even though the latter may be considerably depressed (17). This is also true, in general, of patients with chronic nephritis whose glomerular filtration rate is below normal (18). By contrast, when concentrating capacity (T^cH_2O) was referred to GFR in the present experiments, a considerable difference between normal dogs and those treated with parathyroid extract was apparent. Finally, in two dogs of the present series (3 and 4, Table II) T^cH_2O diminished after parathyroid extract without a concomitant decrease in glomerular filtration rate.

Although a progressive increase in the quantity of solute excreted by each nephron may, under some circumstances, result in the excretion of a dilute urine (19) it seems unlikely that such an increase in solute load was responsible for the present results. Massive solute diuresis in normal dogs did not lower T^cH_2O to the degree observed in animals treated with parathyroid extract. Hypercalcemic dogs of Group I infused with vasopressin produced a hypotonic urine while excreting the same amount of solute per unit of GFR as they had done when they were normal and able to concentrate the urine. Mere destruction of nephron units or an overall decrease in the rate of glomerular filtration could not, therefore, have been primarily responsible for the diminution in concentrating capacity which was observed.

The morphological alterations in these kidneys, localized chiefly to the ascending portion of Henle's loop, the distal convoluted tubule and the collecting ducts, are interesting in the light of current theories concerning the mechanism by which the kidneys reabsorb water and excrete a concentrated urine (20, 21). Impairment of renal concentrating ability might theoretically result from: *1*) a decrease in the reabsorption of sodium without water in the ascending limb of the loop of Henle, *2*) disruption of the counter-current mechanism by which sodium (and urea) are concentrated in the medulla, and *3*) slowing of the back-diffusion of water from the collecting ducts to the hypertonic interstitial fluid of the renal medulla (a process normally facilitated by vasopressin). The data of these experiments are probably compatible with any of the above explanations, although excretion of hypotonic urine by hypercal-

cemic dogs infused with vasopressin implies the continued active reabsorption of sodium without water in distal portions of the nephron.

The changes in concentrating ability observed in these experiments were associated with an elevated serum calcium, yet it is noteworthy that hypercalcemia of the same degree produced acutely by infusions of calcium salts does not result in nearly as large changes in T^cH_2O as those observed here (22). Perhaps the serum level of calcium is less important in this regard than the calcium content of cells lining the medullary tubules, or the structural and metabolic changes induced in the renal medulla by prolonged hypercalcemia and/or hypercalciuria. In addition to producing hypercalcemia, parathyroid hormone probably directly induces certain changes in the metabolism of renal tissue (23) which are not entirely reproduced by increasing the concentration of calcium in the serum, the renal cells, or the urine. It is of interest that morphological and functional changes in the kidneys similar to those of the present experiments can be produced by the administration of large doses of vitamin D to rats (6). The frequent association of polyuria with hypercalcemia and nephrocalcinosis of diverse origins lends support to the thesis that changes in calcium metabolism are at least partially responsible for the hyposthenuria of hyperparathyroidism.

SUMMARY

1. The effects of parathyroid extract upon renal function and structure were studied in dogs.

2. After 24 hours of hypercalcemia induced by parathyroid extract, glomerular filtration rate was usually but not always decreased.

3. Renal concentrating capacity, as measured by the maximum solute concentration of urine during dehydration and by T^cH_2O during mannitol diuresis, was severely impaired. Dogs treated with parathyroid extract excreted a hypotonic urine during moderate mannitol diuresis despite the infusion of large amounts of exogenous vasopressin.

4. These changes were associated with morphological alterations demonstrated by microdissection to lie chiefly in the distal portion of the nephron and the collecting ducts.

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VARIABILITY IN ABSORPTION OF INSULIN-I^{131} IN NORMAL AND DIABETIC SUBJECTS AFTER SUBCUTANEOUS AND INTRAMUSCULAR INJECTION *

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Variability in plasma binding and in peripheral utilization of insulin has been demonstrated in insulin-treated subjects (1, 2). Rates of insulin absorption in diabetics have previously been estimated by noting the insulin effect on the concentration of sugar in the blood, but absorption after subcutaneous and intramuscular injection has not been directly studied in the past. Its importance lies in the possibility that variation in absorption rate attributable to tissue binding or local vascular changes might be one of the factors responsible for alterations in the apparent sensitivity of diabetic patients to insulin.

The present study was undertaken to determine the rate of absorption and its variability in diabetics and in normals and to ascertain whether absorption rates are in any way dependent upon the site of injection. This was accomplished by measuring the disappearance time of insulin-I^{131} from subcutaneous and intramuscular injection sites in 22 diabetic and 11 normal subjects.

MATERIALS AND METHODS

Subjects consisted of hospitalized diabetic patients and volunteer hospital personnel who were apparently in good health. Crystalline beef insulin-I^{131} was obtained from Abbott Laboratories. The lots employed contained an average of one iodine atom per molecule of insulin (molecular weight 6,000). Injections of 0.05 to 0.4 unit of insulin containing 15 to 30 μc. insulin-I^{131} were administered subcutaneously or intramuscularly a total of 92 times to 11 normal and 22 diabetic subjects. The volume of the injected solution ranged from 0.02 to 1.0 ml., adjusted with phosphate buffer (pH 7.39) or, in some instances, with buffer plus nonradioactive (carrier) insulin.

The ages of the diabetic patients ranged from 25 to 70 years and their daily insulin requirements varied from 15 to 70 units. One patient (J.T.) was considered to have "brittle" diabetes and in another (J.M.) the presence of excessive circulating insulin antibodies had been demonstrated one year previously (3). None of the patients showed clinical evidence of lipodystrophy and their diabetes was well controlled during the time of study.

Each experiment was begun at 9 a.m., one and one-half to two hours after daily therapeutic insulin had been given and approximately one hour following breakfast.

Injections were administered in a standardized fashion into the deltoid area of each subject. Usually, simultaneous injections were given in each arm. External monitoring over the injection site was performed immediately and thereafter at hourly intervals for eight hours and in most instances at the end of 24 hours, using a collimated scintillation detector at a distance of 33 cm. With this technique, thyroid radioactivity was excluded and duplicate one minute counts had an average variability of 1.1 ± 1.0 per cent (1 S.D.). Radioactivity at the injection site was then expressed as percentage of initial count. For comparison, the time for absorption of half the radioactivity was used, hereafter designated as $T_{1/2}$.

The *in vitro* stability of the I^{131}-insulin linkage has been demonstrated previously (4, 5, 6). It was assumed that labeling was homogeneous and that disappearance of radioactivity paralleled absorption of intact insulin-I^{131}.

For comparative purposes, NaI^{131} was administered to four of the diabetic subjects and albumin-I^{131} to three additional normal subjects.

RESULTS

Insulin-I^{131} was injected subcutaneously 15 times into 11 normal subjects using two different volumes for injection. One group received a small volume (0.08 to 0.09 ml.), while the other was given an equivalent insulin dose diluted to 0.45 ml. with phosphate buffer. As shown in Figure 1, absorption was approximately exponential in both groups and tended to be more rapid with the larger volume. The mean $T_{1/2}$ for the small-volume group was 213 ± 16 minutes [1]

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[1] This and all following $\pm$ symbols refer to one standard error of the mean.

TABLE I

Variability in insulin-I^{131} absorption in six diabetic patients studied on more than one occasion

Patient	Date	Volume injected		Insulin-I^{131} per injection	Subcutaneous $T_{\frac{1}{2}}$		Intramuscular $T_{\frac{1}{2}}$	
		Right arm	Left arm		Right arm	Left arm	Right arm	Left arm
		ml.	*ml.*	*μc.*	*min.*	*min.*	*min.*	*min.*
A. C.	Jan. 14	0.15	0.15	22	261	348		
	Jan. 25	0.03	0.03	23	160			108
	Feb. 11	0.45	0.45	15		127	137	
	Feb. 14	1.00	0.30	17	110	110		
F. S.	Jan. 25		0.28	22		305		
	Feb. 14	0.80	0.20	17	98	94		
	June 26	0.51		10	181			
G. C.	Jan. 28	0.05	0.05	29		23½ hrs.	155	
	Jan. 31	0.02		9	211			
G. S.	Jan. 28	0.05	0.05	28	186	144		
	Feb. 11	0.45	0.45	15		126	118	
J. T.	Jan. 15	0.05	0.05	19	88	123		
	Jan. 30	0.03	0.03	23		84	140	
	Feb. 12	0.45	0.45	17		78	95	
	Feb. 15		0.45	17		83		
	May 31	0.45	0.45	12	53	71		
E. D.	Dec. 10	0.03	0.03	20	160	16 hrs.		
	Jan. 25	0.03	0.03	23	190			145
	Feb. 15	0.45	0.45	17		16½ hrs.	54	

and was significantly greater than the mean of 130 ± 16 minutes in the large-volume group ($p < 0.01$).

Twenty-two diabetic patients were similarly given a total of 50 injections, using volume schedules similar to those employed in the normal group (Figure 1). A total volume of 0.02 to 0.30 ml. was injected subcutaneously 18 times into 10 diabetics and volumes of 0.45 to 1.0 ml., containing comparable μc. concentrations, were administered 32 times to 19 diabetics. As in the normal subject, disappearance of radioactivity tended to be more rapid in diabetics when the larger volumes were employed. The mean $T_{1/2}$ of 173 ± 18 minutes in the small-volume group was not significantly greater than the mean of 146 ± 12 minutes in the large-volume group, however ($p > 0.1$). Individual variation in absorption rate was greater in diabetics, with a range in $T_{1/2}$ of 53 to 348 minutes as compared to 90 to 282 minutes in the normal group. Absorption in a given patient appeared to vary considerably on different occasions and to vary from arm to arm with simultaneous injection (Table I).

Markedly delayed absorption was demonstrated in two diabetic patients, omitted from the above means. In one of these the $T_{1/2}$ was approximately 23.5 hours, while in the other it was slightly over 16 hours on two occasions. In each instance, however, a normal rate of disappearance was observed simultaneously in the contralateral arm (Table I). The patient with "brittle" diabetes had consistent but rapid absorption rates (Table I). The patient with previously demonstrated insulin resistance (J.M.) had a modal absorption half-time.

Using comparable volumes for injection and omitting the two patients with markedly prolonged absorption, no significant difference could be demonstrated between normals and diabetics. The mean $T_{1/2}$ in the normal, regardless of the volume injected, was 160 ± 17 minutes as compared to 156 ± 10 minutes in the diabetic. No correlations could be found between absorption rate and insulin requirement, body weight, duration of the disease or specific activity (age) of the insulin employed.

In an effort to investigate possible sources of the observed variability, individual variations in subcutaneous absorption rate were studied by the

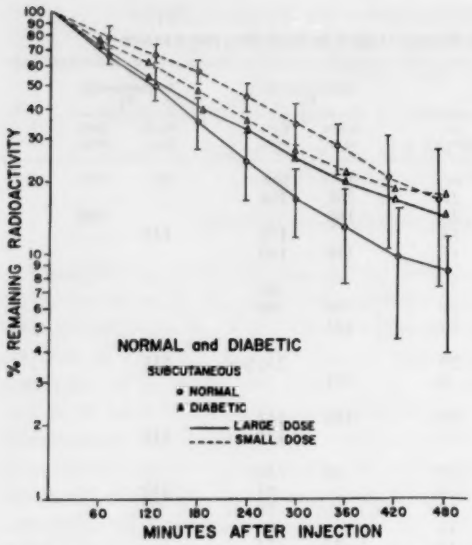

FIG. 1. COMPARISON OF ABSORPTION FROM SUBCUTANEOUS SITES IN NORMALS AND DIABETICS ACCORDING TO VOLUME INJECTED

Means ± 2 S. E. for normal groups are represented by circles and means for diabetic groups by triangles. Large volumes (0.45 to 1.0 ml.) are represented by solid lines; small volumes (0.02 to 0.30 ml.) by broken lines. Absorption half-times of more than 16 hours, found on three occasions in two patients, are omitted from these and subsequent curves and averages.

simultaneous administration of equal insulin doses into both arms in four normal and 12 diabetic subjects. In the normal group, the mean absolute difference in absorption half-times between arms for the simultaneous injections was 44 minutes (range of differences, 40 to 47 minutes). In the diabetic group it was 34 minutes (range of differences, five to 87 minutes). Although the range of differences between arms was greater in the diabetics, the average of these differences did not vary significantly from that of the normal group.

Simultaneous intramuscular and subcutaneous injections were given to seven normal and five diabetic subjects. Results are shown in Figure 2. In both normals and diabetics, absorption half-times were somewhat less after intramuscular injection and there was also less individual variation by this route. The mean $T_{1/2}$ in the normal group was 130 ± 17 minutes subcutaneously and 88 ± 7 minutes intramuscularly, as compared to 142 ± 19 minutes and 123 ± 6 minutes, respectively, in the diabetic group. No significant difference was noted between subcutaneous and intramuscular absorption half-times in either group ($p > 0.05$). The mean intramuscular $T_{1/2}$ of 123 minutes in the diabetic group was significantly greater than the mean of 88 minutes in the normal, however ($p < 0.01$). "Tailing" of the intramuscular curves after four hours was noted in both groups.

In eight diabetics, a subcutaneously administered tracer dose diluted with phosphate buffer was compared with a simultaneous injection in which 10 to 20 units of nonradioactive (carrier) insulin with added phosphate buffer was used as diluent (Figure 3). Although the addition of carrier prolonged the mean $T_{1/2}$ from 191 ± 26 minutes to 240 ± 22 minutes (mean difference, 49 ± 18 minutes, $p < 0.05$), this was largely due to delayed absorption in the first two hours and the configuration of the absorption curves was similar thereafter.

The amount of radioactivity remaining at the in-

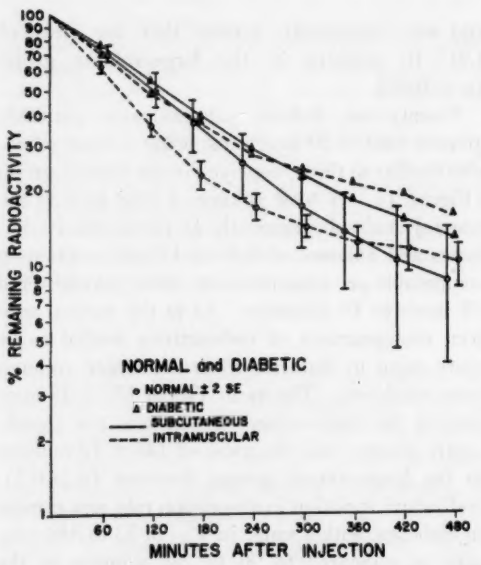

FIG. 2. COMPARISON OF SUBCUTANEOUS AND INTRAMUSCULAR ABSORPTION IN NORMALS AND DIABETICS

Means ± 2 S. E. for normal groups are represented by circles; means for diabetic groups by triangles. Subcutaneous routes are represented by solid lines; intramuscular routes by broken lines.

jection site after 24 hours was determined in five normal and six diabetic subjects. In the normal group, a mean of 1.5 ± 0.4 per cent of initial radioactivity remained following subcutaneous injection. This was significantly less than the mean of 4.1 ± 0.9 per cent noted after intramuscular injection ($p < 0.01$). In the diabetic group, means of 2.5 ± 0.3 per cent and 8.2 ± 1.8 per cent remained at the respective subcutaneous and intramuscular sites after 24 hours. This difference was also significant ($p < 0.02$). The amount of radioactivity remaining at subcutaneous sites was not significantly different in normals and diabetics ($p > 0.05$), but the mean of 4.1 per cent noted at intramuscular sites in the normal was significantly less than the mean of 8.2 per cent noted in the diabetic group ($p < 0.05$).

For comparative purposes, four diabetic patients were given simultaneous subcutaneous and intramuscular injections of NaI^{131} (Figure 4). Absorption was rapid and virtually complete within one hour. It was slightly faster intramuscularly (mean $T_{1/2} = 20$ minutes) than subcutaneously (mean $T_{1/2} = 25$ minutes), but the difference was not significant.

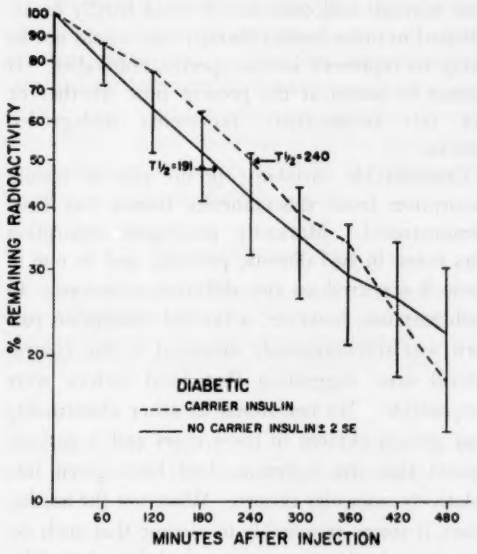

FIG. 3. EFFECT OF DILUTION OF SUBCUTANEOUSLY ADMINISTERED TRACER INSULIN-I^{131} WITH NONRADIOACTIVE CARRIER INSULIN

Mean ± 2 S. E. without carrier is represented by solid line; with added carrier, by broken line.

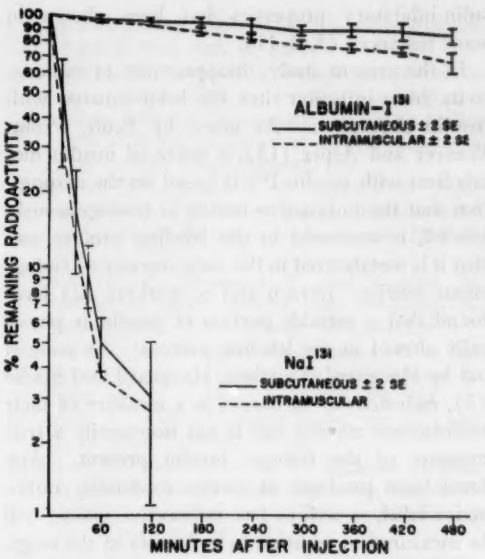

FIG. 4. SUBCUTANEOUS AND INTRAMUSCULAR ABSORPTION OF ALBUMIN-I^{131} IN THREE NORMAL SUBJECTS AND OF NaI^{131} IN FOUR DIABETICS

Means ± 2 S. E. for subcutaneous routes are represented by solid lines; intramuscular routes by broken lines.

Albumin-I^{131} was administered subcutaneously and intramuscularly to three normal subjects (Figure 4). Absorption was considerably slower than it was with insulin-I^{131}. After two hours, disappearance of radioactivity from the intramuscular site was significantly faster than it was from the subcutaneous site ($p < 0.01$), and by extrapolation, $T_{1/2}$ was estimated to be approximately 13 and 22 hours by the respective routes.

DISCUSSION

The presence of insulin-binding moieties in the circulating plasma proteins of insulin-treated subjects is well documented (1–3, 7–14). These moieties have been considered to be antibodies (2, 11–13) and usually have been found to be associated with the gamma and beta globulin fractions of the plasma proteins (2, 3, 9, 10–13). A correlation between insulin requirement and serum in-

sulin-inhibitory properties has been shown in some instances (3, 9, 11).

In the present study, disappearance of radioactivity from injection sites has been equated with insulin absorption. As noted by Scott, Prout, Weaver and Asper (15), a study of insulin metabolism with insulin-I^{131} is based on the assumption that the radioactive insulin is homogeneously labeled, is unaltered in the labeling process and that it is metabolized in the same manner as endogenous insulin. Berson and co-workers (2) have found that a variable portion of insulin is physically altered in the labeling process. As pointed out by Haugaard, Vaughan, Haugaard and Stadie (5), radioactivity in tissues is a measure of their radioisotope content and is not necessarily a true measure of the isotopic insulin present. Any breakdown products of insulin containing radioactive label, as well as free radioactive iodine, will be measured in addition to molecules of the originally injected insulin. For comparative purposes, however, these factors would not appear to be of major consequence. The persistence of significant amounts of free I^{131} at the site of injection seems unlikely in view of the rapid absorption demonstrated with NaI^{131}.

If local breakdown of iodinated insulin occurs and is enzyme-dependent (*i.e.*, not random), one would expect a retardation in disappearance of the label once the maximum capabilities of the enzyme system were exceeded. It was shown, however, that dilution of labeled insulin with phosphate buffer tended to hasten absorption in both normals and diabetics, perhaps related to the higher pH in the buffered system or to an easier access to the bloodstream. Addition of nonradioactive carrier insulin somewhat prolonged the disappearance time of the label.

In the present study, no significant difference between subcutaneous and intramuscular absorption half-times could be demonstrated in either normals or diabetics. When diabetics were compared with normals, subcutaneous absorption rates were found to be similar, although intramuscular absorption was significantly faster in the normal group.

Berson and co-workers (2) have suggested that fluctuations in the production of "insulin transporting" antibodies may be causally related to the lability of insulin requirements in those patients who manifest what has been termed "brittle" diabetes. It is also possible that these or other antibodies may be present locally at the site of insulin injection, either cell-bound or free in the extracellular fluid. Since the above authors (2) found maximum plasma binding in nonresistant patients to be in the range of only a few units per L. of plasma, the total amount of antibody at an injection site would probably be small in relation to the doses of insulin employed therapeutically or in the present study (0.05 to 0.4 unit). The similar absorption half-times in normals and diabetics following subcutaneous injections would indicate that local insulin antibodies, if present, are probably of no therapeutic significance.

The fact that the mean intramuscular $T_{1/2}$ in diabetics was significantly greater than in the normals, coupled with the finding of significantly greater radioactivity after 24 hours, could be interpreted as evidence for "tissue binding." The tailing of the intramuscular curves also indicates some binding at that site which is not present in the subcutaneous tissues. Since this was noted in both normals and diabetics, it could hardly be attributed to prior insulin therapy and would not be likely to represent insulin-specific antibodies. It cannot be stated at the present time whether or not this radioactivity represents undegraded insulin.

Considerable variability in the rate of insulin absorption from subcutaneous tissues has been demonstrated. Markedly prolonged absorption was noted in two diabetic patients, and in one of these it occurred on two different occasions. In each instance, however, a normal absorption pattern was simultaneously observed in the contralateral arm, suggesting that local factors were responsible. No hematoma or other abnormality was grossly evident in these cases and it was assumed that the injections had been given into relatively avascular tissues. Whatever the mechanism, it seems reasonable to assume that such occurrences in the therapeutic administration of insulin might account, in part at least, for fluctuations in the control of some diabetic patients.

SUMMARY AND CONCLUSIONS

1. Insulin-I^{131} was administered subcutaneously or intramuscularly to 11 normal and 22 diabetic subjects and rates of disappearance of radioactivity from the injection sites were determined.

2. Absorption was approximately exponential but there was marked variation in rates of disappearance from subcutaneous tissues. Less variation was noted following intramuscular injection.

3. No significant difference between subcutaneous and intramuscular absorption half-times was found in either normals or diabetics.

4. Intramuscular absorption was significantly faster in normals than in diabetics and at 24 hours the residual radioactivity at intramuscular sites was significantly less in the normals.

5. "Tailing" of intramuscular curves was noted in both normals and diabetics and suggested some tissue binding in that site which was not present in the subcutaneous tissues.

6. Dilution of the administered tracer dose with phosphate buffer shortened mean half-times. The addition of carrier prolonged absorption somewhat, but results were inconclusive.

7. Extremely prolonged absorption was demonstrated in two diabetic patients, in each of whom a normal disappearance rate was simultaneously observed in the contralateral arm, suggesting that local factors, perhaps relative avascularity, were responsible. Such occurrences in the therapeutic situation may account in part for fluctuations in the control of some diabetic patients.

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HEMATOGENOUS PYELONEPHRITIS IN RATS. IV. RELATIONSHIP OF BACTERIAL SPECIES TO THE PATHOGENESIS AND SEQUELAE OF CHRONIC PYELONEPHRITIS * †

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Despite the inherent differences in the various bacterial species responsible for human pyelonephritis, little consideration has been given to differences in the characteristics of the renal disease resulting from infection by each species. While it is possible that pyelonephritis is a single disease entity that develops in an identical fashion regardless of the type of bacteria, scattered clinical observations suggest that variations in the pathologic processes are governed to some extent by the properties of the infecting micro-organisms (1–4).

In order to obtain further information on the pathologic differences resulting from renal infection by a variety of bacterial species, pyelonephritis was produced in rats by a method that allows the infection to develop without the confusing effects of surgical hydronephrosis (5). In the study described in the preceding paper in this series, remarkable differences were observed in the character of the acute and subacute renal infection produced by various strains of *Escherichia coli, Proteus, Pseudomonas aeruginosa* and enterococci isolated from patients with pyelonephritis (6). The present study was designed to examine the differential pathological effects of three of these species in chronic pyelonephritis in rats which were followed as long as one year after the onset of renal infection. In addition, the relationship of chronic pyelonephritis to hypertensive vascular disease was studied in an attempt to learn whether differences in bacterial species could account for the inconstant occurrence of hypertension in human chronic pyelonephritis and its absence in experimental chronic pyelonephritis due to *E. coli* (7).

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MATERIALS AND METHODS

Female albino rats of the Holtzman strain weighing 200 to 250 Gm. were used in all studies. Pyelonephritis was established by intracardiac inoculation of either *E. coli, Proteus morganii* or *Streptococcus zymogenes* and massage of the kidney in the manner described previously (5). Each bacterial strain was initially recovered from the urine or blood of patients with pyelonephritis and after growth for 18 hours was frozen and stored in trypticase soy broth at $-22°$ C. Immediate mortality, within the first 48 hours after inoculation was approximately 50 per cent in animals infected with *E. coli* or *P. morganii*, but was absent after *S. zymogenes*. One hundred rats survived initial infection with *E. coli*, 62 rats with *P. morganii* and 73 rats with *S. zymogenes*. Animals infected with each species were then divided into groups which received a second, third or fourth inoculation of the same organism at 10 to 20 week intervals, as indicated in Tables IA through C. Each reinoculation was accompanied by renal massage. Several animals from each subgroup were sacrificed between each inoculation and the remainder approximately one year after the initial infection. A group of 40 control rats were included in the study; 12 of these were sacrificed midway during the experiment and the remaining 28 followed for up to one year (Table ID). All animals were housed in groups of six to 12 in colony cages, fed identical diets of rat chow and allowed tap water for drinking *ad libitum*.

As previously described, the kidneys of each rat were gently but firmly massaged during ether anesthesia for exactly five minutes. In order to minimize subjective factors that might influence the intensity of renal massage, each period of five minutes was divided between any two of us instead of confining the total period for each animal to one person. The kidneys of control animals were not massaged, since it had been demonstrated previously that massage alone does not produce permanent injury (7).

Bacteriological and pathological study of the kidneys and blood cultures were carried out in every rat with the techniques described elsewhere (5–7). Cultures were also made of portions of liver and spleen. All tissues were ground to a pulp with sterile sand, suspended in water and spread across a blood agar plate for culture. Sections for histologic study were made through the kidney

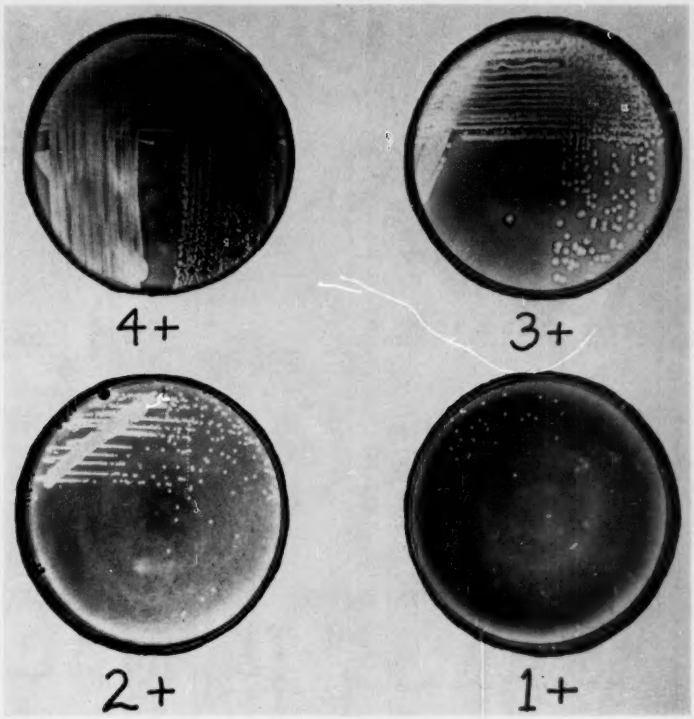

FIG. 1A. SYSTEM FOR GRADING INTENSITY OF RENAL INFECTION FROM GROWTH ON BLOOD AGAR PLATES

at the hilum so that the entire cortex, medulla and pelvis were examined at essentially the same planes in all specimens. The rats were weighed and their systolic blood pressures were determined monthly throughout the study, the latter by the tail plethysmographic method as reported previously (7).

Impairment of renal function was investigated by determining the blood urea nitrogen (BUN) of samples collected by intracardiac puncture upon sacrifice and by measuring the ability of the animals to concentrate urine during the course of the experiment. For the latter purpose the rats were placed in metabolic cages without food or water for 48 hours and the urine collected quantitatively in graduates which contained mineral oil and a small amount of phenylmercuric nitrate. The osmolal concentrations of the urine samples collected between 24 and 48 hours were determined by the freezing point technique with a Fiske osmometer.

RESULTS

1. Mortality

Although the immediate mortality after the first injections with *E. coli* and *P. morganii* was approximately 50 per cent, the subsequent combined acute mortality from repeated inoculations of these bacteria was less than 10 per cent. This decrease was attributed to the development of resistance to the endotoxins of the Gram-negative bacteria. The Gram-positive organism, *S. zymogenes,* neither killed the rats nor made them outwardly ill. Aside from the initial inoculation of Gram-negative bacteria, a major cause of death during the course of the study was pneumonia which affected all groups equally, including the controls. Most of these deaths occurred during the last three months of the experiment and were due to Type 2 pneumococci and other primary pulmonary pathogens, but not the bacteria inoculated experimentally. An additional 12 per cent of the rats were killed accidentally.

2. Bacteriological findings

Among the rats inoculated with *E. coli,* kidneys of all but four of 31 animals sacrificed 14 weeks or

TABLE 1A

Rats infected with E. coli

Group (no. of injections)	No. of rats	Time of sacrifice: Weeks after 1st infection	Weeks after 2nd	Weeks after 3rd	Weeks after 4th	Renal function: Positive kidney cultures (no. of rats)	Infected kidneys with sterile urine (no. of rats)	BUN	Osmolar concentration (wk. of expt.)	Systolic blood pressure: Final BP	No. of rats above 140 mm. Hg	Average elevation above 110 mm. Hg	Heart weight
								mg./100 ml.	*mOsm./Kg.*	*mm. Hg*			*mg./100 Gm.*
E (1)	2	8				1	0	25		98	0	−10	
	2	38				0	0	27		105	0	− 8	
	6*	55				0	0	29	2,660 (33)	124	0	+ 6	274
E (2)	2	9	1 da.			2	0	45		95	0	−15	
	7	10	1			7	2	34		94	0	−11	
	2	22	12			0	0	22		110	0	− 4	
	2	34	25			1	0	28		103	0	− 4	
	6*	56	47			0	0	32	2,798 (39)	117	0	0	277
E (3)	2	20	10	1 da.		2	0	44		115	0	− 3	
	2	23	14	1		2	1	24		105	0	− 5	
	2	29	20	10		0	0	25		100	0	0	
	8*	53	43	33		2	0	28	3,009 (29)	127	1	+ 7	282
E (4)	7*	50	41	33	25	1	1	29	3,106 (33)	119	0	+ 3	270

* Sacrificed at end of study.

TABLE 1B

Rats infected with Proteus morganii

Group (no. of injections)	No. of rats	Time of sacrifice: Weeks after 1st infection	Weeks after 2nd	Weeks after 3rd	Renal function: Positive kidney cultures (no. of rats)	Infected kidneys with sterile urine (no. of rats)	Renal stones (no. of rats)	BUN	Osmolar concentration (wk. of expt.)	Systolic blood pressure: Final BP	No. of rats above 140 mm. Hg	Average elevation above 110 mm. Hg	Heart weight
								mg./100 ml.	*mOsm./Kg.*	*mm. Hg*			*mg./100 Gm.*
P (1)	2	13			2	0	2	31		125	1	+10	
	3	24			3	1	1	41		123	0	+12	
	14*	51			8	2	2	35	2,514 (20)	121	0	+13	281
P (2)	2	16	1 da.		2	0	0	50		115	0	+10	
	2	24	3 da.		2	0	0	25		125	0	+14	
	3	17	1		3	0	0	29		105	0	+10	
	2	34	18		2	1	1	32		133	1	+13	
	13*	48	24		10	2	1	33		122	1	+14	283
P (3)	4*	53	37	15	4	1	2	31	2,473 (47)	110	0	+ 1	294

* Sacrificed at end of study.

TABLE IC

Rats infected with Streptococcus zymogenes

Group (no. of injections)	No. of rats	Time of sacrifice: Weeks after 1st infection	Weeks after 2nd	Weeks after 3rd	Renal function: Positive kidney cultures (no. of rats)	Infected kidneys with sterile urine (no. of rats)	BUN	Osmolar concentration (wk. of expt.)	Systolic blood pressure: Final BP	No. of rats above 140 mm. Hg	Average elevation above 110 mm. Hg	Heart weight
							mg./100 ml.	*mOsm./Kg.*	*mm. Hg*			*mg./100 Gm.*
S (1)	2	16			2	0	23		123	0	+ 9	
	4	22			4	0	27		117	0	+12	
	8*	44			8	1	24	2,176 (30)	120	0	+11	283
S (2)	3	15	1 da.		3	0	26		113	0	+ 5	
	2	16	1		2	0	26		98	0	+ 8	
	2	24	9		2	0	26		110	0	+ 2	
	2	44	19		2	1	29		128	0	+ 2	
	6*	54	28		6	0	25	2,323 (40)	127	1	+12	274
S (3)	2	24	9	3 da.	2	0	26		115	0	+ 4	
	2	43	28	19	2	0	28		123	0	+14	
	7*	53	38	28	7	0	25	1,957 (40)	118	0	+ 8	272

* Sacrificed at end of study.

TABLE ID

Control group

Group	No. of rats	Time of sacrifice (wks. after start of study)	Renal function: Positive kidney cultures (no. of rats)	BUN	Osmolar concentration (wk. of expt.)	Systolic blood pressure: Final blood pressure	No. of rats above 140 mm. Hg	Average elevation above 110 mm. Hg	Heart weight
				mg./100 ml.	*mOsm./Kg.*	*mm. Hg*			*mg./100 Gm.*
K-1	12	24	0	24		113	0	+3.0	275
K-2*	11	56	0	25	3,827 (34)	116	0	+6.0	279

* Sacrificed at end of study.

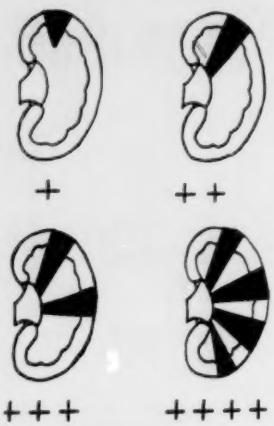

FIG. 1B. SYSTEM FOR GRADING SEVERITY OF GROSS LESIONS IN KIDNEYS WITH CHRONIC PYELONEPHRITIS

longer after their final injection had become sterile. The occasional positive cultures were obtained only from animals receiving two or more inoculations. On the other hand, infection with *S. zymogenes* persisted in the kidneys of all animals, up to the final sacrifice 44 weeks after one or more injections. Infection with *Proteus* was also persistent; up to 24 weeks, kidneys were positive in almost 100 per cent of the rats, while the kidneys in eight of 14 animals receiving only one injection were still infected at the end of a year (Tables IA through D).

Quantitative cultures of the kidneys were performed as previously described (5) and the results recorded according to the scheme in Figure 1A. In Table II, the intensity of the infection in the kidneys obtained from the rats sacrificed at the termination of the experiment are compared for the three species. These data indicate that the infection of the kidneys with enterococci not only persisted longer but that the numbers of bacteria in the kidneys infected with this species also were most numerous.

Neither *E. coli* nor *P. morganii* could be cultured from the blood of animals sacificed one week or later after inoculation or reinoculation of bacteria. With the exceptions of two rats, it was also impossible to isolate *S. zymogenes* from the blood of the 35 pyelonephritic animals examined at any time after the first week of inoculation or reinoculation with that organism. Hence the strongly positive cultures of kidneys from rats with streptococcal or *Proteus* pyelonephritis could not be attributed to bacteremia and the presence of infected blood in the kidneys. Many of the animals with *Proteus* or streptococcal pyelonephritis yielded on culture a few colonies of these bacteria in their liver or spleen. These slightly positive cultures of extrarenal tissue were considered to be secondary to the persistent renal infection, rather than their cause for the following reasons: *1*) Bacteria could be cultured from extrarenal tissues in only 76 per cent of the animals with infected kidneys and never in the absence of renal infection; *2*) only an occasional bacterial colony (less than one in Figure 1A) was recovered

TABLE II

Comparison of intensity of infection in rats sacrificed terminally

Species	No. of injections	No. of kidneys examined	Intensity of infection					"Intensity index"*
			0	1+	2+	3+	4+	
E. coli	1	12	12	0	0	0	0	0
	2	11	10†	0	0	0	0	0
	3	16	13	2	0	1	0	0.08
	4	14	13	1	0	0	0	0.03
P. morganii	1	28	14	5	4	5	0	0.25
	2	26	9	9	8	0	0	0.24
	3	8	0	3	1	4	0	0.53
S. zymogenes	1	16	1	2	4	8	1	0.59
	2	12	0	0	4	2	6	0.79
	3	14	0	1	3	4	6	0.77

* "Intensity index" $= \frac{\text{sum of grades}}{\text{number of kidneys examined} \times 4}$.

† One kidney absent, one kidney infected with *Proteus*.

from extrarenal tissues despite heavy renal infection; *3*) there were no pathologic changes in the liver or spleen while the infected kidneys showed severe inflammatory changes.

In view of the clinical dependence on positive urine cultures to establish the diagnosis of pyelonephritis, it is of interest that sterile urine or bladder cultures were obtained from 13 animals possessing infected kidneys. The renal infection was due to *E. coli* in four of these animals, to *Proteus* in seven and to enterococci in two (Tables IA through C).

3. Blood pressures and heart weights

In Tables IA through D, blood pressure data are expressed in several ways. The mean of the final blood pressure determinations for the rats in each group are listed as well as the number of animals with systolic pressures of 140 mm. Hg or higher. In addition, individual and average monthly deviations from 110 mm. Hg [1] were calculated for each animal, and the means for the groups were determined.

Significant hypertension failed to develop as indicated by the rare occurrence of blood pressure over 140 mm. Hg. Analysis of variance of the blood pressures in animals sacrificed terminally demonstrate that although hypertension did not develop and the final pressures among the various groups were not different, significant elevations above 110 mm. Hg, as compared with the control group of equal age, occurred in the animals inoculated once or twice with *Proteus* ($p = 0.01$, Table III). These small changes in blood pressure in the *Proteus* groups, however, did not lead to a significant increase in heart weight (Table III).

It was not possible to correlate severity of renal involvement or differences in the damage between the two kidneys with the level of blood pressure. However, the highest individual pressure (155 mm. Hg) was found in an animal which at autopsy demonstrated a tiny, atrophic right kidney and a hypertrophied and deformed left kidney. This animal was injected three times with *E. coli* and died spontaneously 12 weeks after the third and 32 weeks after the first inoculation.

In view of the possibility that early deaths may have occurred in those rats with the severest renal disease and that hypertension may have con-

[1] The average systolic blood pressure of a large series of normal young rats in our laboratory is 110 ± 10 mm. Hg with a further rise of approximately 10 mm. Hg with age. Spontaneous pressures of 140 mm. Hg or above are rare in our experience and in those of others employing the same technique (8).

The calculation of "average elevation in blood pressure over 110 mm. Hg" takes into account changes that occur during the entire course of the experiment. It avoids the possibly misleading impression obtained from single terminal determinations in sick hypertensive animals which may develop a fall in blood pressure before death or sacrifice. Normal adult rats with an average elevation of more than + 15 mm. Hg during their lifetime are rare.

TABLE III

Analysis of variance data from animals sacrificed at termination of study

Group	Final blood pressure		Average BP above 110 mm. Hg		Heart weight		BUN		Osmolal concentration	
	Mean	± S. D.	Mean	± S. D.	Mean	± S. D.	Mean	± S. D.	Mean	± S. D.
K-2	115.9	8.6	6.3	5.5	279.2	16.8	24.8	3.3	3,827	837
E (1)	123.8	13.5	6.2	5.4	273.6	18.5	29.2	5.0	2,660*	633
E (2)	116.7	10.8	−0.3*	5.7	277.4	22.2	32.4	9.4	2,798*	387
E (3)	126.9	12.2	7.1	3.6	281.8	11.3	28.0	3.5	3,009†	959
E (4)	119.3	9.3	3.3	6.9	269.5	22.0	28.6	5.2	3,106	592
P (1)	121.4	7.5	12.9*	4.8	280.5	23.4	35.0*	13.2	2,515*	933
P (2)	122.3	6.3	13.5*	5.4	282.8	22.4	32.2*	11.5		
P (3)	110.0	9.1	1.0	6.3	293.7	33.0	30.5	7.9	2,473*	1,000
S (1)	120.0	13.1	10.8	6.3	283.3	24.1	23.9	3.1	2,176*	400
S (2)	127.4	8.8	12.0	5.7	273.5	28.3	25.0	3.0	2,323*	633
S (3)	117.9	6.3	8.4	5.6	272.0	14.7	25.4	2.5	1,957*	412
F	1.53	$p > 0.05$	6.68	$p < 0.01$‡	0.48	$p > 0.05$	2.15	$p = 0.05$‡	4.28	$p < 0.01$‡

* Significant differences from K-2 group; $p = 0.01$ or less.
† Significant difference from K-2 group; $p = 0.05$.
‡ Significant F values.

TABLE IV

Blood pressure in rats which died spontaneously during study

Group	No. of rats	Average time of death: Weeks after 1st infection	Weeks after 2nd	Weeks after 3rd	Weeks after 4th	Systolic blood pressure: Final BP	No. of rats above 140 mm. Hg	Average elevation above 110 mm. Hg
						mm. Hg		
K-2	17	31				119	1	+ 7
E (1)	21	24				115	1	+ 1
E (2)	17	23	14			106	0	− 4
E (3)	9	28	18	9		117	1*	+ 2
E (4)	3	26	17	9	5 da.	117	0	+ 6
P (1)	5	45				120	0	+13
P (2)	9	41	19			117	0	+13
P (3)	3	48	33	10		115	0	+10
S (1)	12	26				116	0	+11
S (2)	14	36	15			121	0	+ 7
S (3)	7	48	32	22		120	1	+12

* Maximum blood pressure in this rat was 155 mm. Hg (see text).

tributed to their demise, similar data were calculated for rats dying spontaneously (Table IV). The blood pressure changes were identical; hypertension failed to develop, but higher blood pressures were indicated in the groups with *Proteus* infection.

4. Pathologic findings

The changes of chronic pyelonephritis were present in every rat sacrificed at the termination of the experiment approximately one year after initial infection and were observed as early as 24 weeks. The kidneys of animals sacrificed at the end of the experiment were graded on a scale of 1 + or 4 + according to the severity of gross pyelonephritic changes (Figure 1B and Table V). Gradings were done independently by two different observers and the results were averaged. As in the case of acute pyelonephritis (6), the most severe destruction in the chronic stage resulted from infections with *Proteus*. A striking discrepancy was noted again between renal damage and intensity of infection, when the *E. coli* groups were compared with those infected by enterococci. *E. coli* had disappeared from nearly every infected kidney by the end of one year but the renal damage observed at this time was as great or greater than that in kidneys still heavily infected with enterococci. It is of interest that repeated inoculation usually failed to increase the severity of the lesions.

TABLE V

Comparison of severity of chronic pyelonephritis approximately one year after infection by different bacterial species

Species	No. of injections	No. of kidneys examined	Grade of severity: 1+	2+	3+	4+	"Severity index"*
E. coli	1	12	2	3	3	4	0.69
	2	11	2	3	0	6	0.73
	3	16	6	3	4	3	0.56
	4	14	4	3	5	2	0.59
P. morganii	1	28	4	6	9	9	0.71
	2	26	2	3	8	13	0.83
	3	8	0	2	1	5	0.84
S. zymogenes	1	16	2	6	6	2	0.56
	2	12	2	8	2	0	0.50
	3	14	1	9	4	0	0.55

* "Severity index" = $\frac{\text{sum of grades}}{\text{number of kidneys examined} \times 4}$.

There was also a remarkable difference in the distribution of lesions produced by the different species (Figures 2A through C). In rats infected with enterococci, cortical lesions were observed microscopically in only 57 per cent of the kidneys but damage to the medulla and pelvic mucosa was invariably present and the severity usually approximated that seen in infections by *E. coli* and *Proteus*. The Gram-negative bacilli, on the other hand, produced lesions in the cortex of almost every kidney (*E. coli* 96 per cent, *Proteus* 100 per cent) but not always in the medulla (*E. coli* 82 per cent, *Proteus* 93 per cent).

Regardless of bacterial etiology, all animals exhibited at least some of the characteristic changes observed in chronic pyelonephritis in man (Fig-

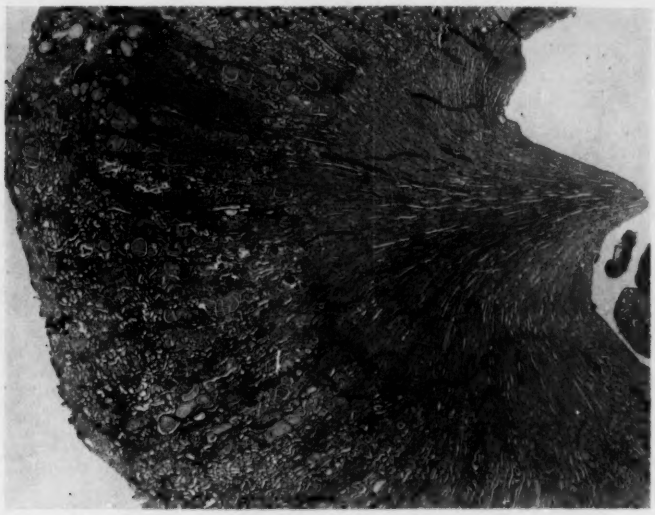

FIG. 2A. CROSS SECTION OF KIDNEY INFECTED WITH *E. COLI* (× 15)
Note diffuseness of lesions affecting cortex and medulla.

ures 3 through 6). Kidneys were distorted, lobulated by scars, adhered to surrounding organs and resistant to section. Periglomerular fibrosis was prominent but the glomeruli themselves appeared normal unless destroyed by suppuration. The tubules were distorted, dilated and frequently filled with colloid casts. Interstitial fibrosis and infiltration of mononuclear cells were marked. The renal papillae were thickened and irregular so that on cross section the mucosa appeared to invade the papillae and to produce "polypoid masses" composed of pelvic epithelium and granulation tissue. Occasional pus casts were noted but almost exclusively in infected kidneys.

Kidney stones were found in nine of the 45 animals with *Proteus* infection (20 per cent). These ranged from small concentrations of gravel found near the papillary tip to large, classical "staghorn calculi" filling the entire pelvis of the kidney. Hydronephrosis was noted in association with the stones.

Vascular changes were minimal in all groups. Medial hypertrophy was detected only rarely and was slight. In areas where destruction of renal parenchyma and fibrosis were marked, there was some hyalinization of the media. These changes were most outstanding in the animals infected with *P. morganii*. Intimal thickening was absent and the ischemic type of vascular lesion described in man by Kincaid-Smith was not observed (9).

5. Renal function

The serial data in Tables 1A through C indicate that azotemia occurred immediately after reinfection in the groups infected with *E. coli* or *Proteus* but then tended to subside. A significant elevation of BUN was noted again at the termination of the experiment in two of the groups infected with *P. morganii* ($p = 0.01$, Table III). No elevation of BUN was found in any of the rats infected with enterococci. The group means in the animals infected with *E. coli* likewise were not significantly elevated from normal, but the large standard deviations indicate that some individual animals were azotemic (Table III).

Tables IA through D and Table III also indicate that the osmolal concentration of urine collected after dehydration was reduced below normal in all groups except, paradoxically, those rats receiving four inoculations of *E. coli*. With all of the species, repeated inoculation did not lower the osmolal concentration below that of rats given only one inoculation. It is of interest that osmolal concentration was impaired equally in groups infected with *Proteus* and enterococcus even though

FIG. 2B. CROSS SECTION OF KIDNEY INFECTED WITH *P. MORGANII* (×15)
Lesions are somewhat more localized than in (A) but all parts of the kidney are affected.

azotemia and damage to renal tissue were greater in those infected with *Proteus*. This suggests that with certain bacterial species hyposthenuria may be a more sensitive indicator of impaired renal function in pyelonephritis than azotemia.

DISCUSSION

The different features of pyelonephritis that were attributed in the acute stage to differences in the bacterial species persisted and sometimes progressed as the infections were traced into the chronic stage. As late as one year after the initial infection, *Proteus* remained the sole cause of renal stones and still produced the greatest renal damage. Animals infected with enterococci, on the other hand, continued to show milder renal damage even though their kidneys remained infected with the most bacteria. In chronic *E. coli* pyelonephritis, the discrepancy between renal damage and infection was again the reverse of that with enterococci, and now even more marked than during the acute stage; *E. coli* had vanished from almost every kidney, although the renal damage was extensive and as great or greater than with the enterococcus.

With the passage of time, additional changes were found that were absent in the acute stage. In all three types of infection, the typical histologic features of chronic pyelonephritis became prominent. While the nature of these chronic changes were fundamentally the same for each of the three bacterial species, an important difference was noted between the distribution of lesions in infections by Gram-negative and Gram-positive bacteria. The Gram-negative bacteria produced both cortical and medullary involvement in virtually every kidney, while in chronic enterococcal infections almost 50 per cent of the kidneys were free of cortical lesions despite their invariable development of medullary and pelvic lesions. It was surprising to find that repeated bacterial inoculation did not increase the tissue damage of the kidney or further reduce their function. The first infection produced an obvious increase in resistance to the systemic effects of Gram-negative bacilli, so that mortality from bacteremic shock became negligible after subsequent inoculations. Studies are now in progress to determine whether prior infection also evoked a state of local resis-

tance in the kidney itself through the development of tolerance to the bacterial endotoxins.

The absence of significant vascular disease and hypertension in experimental chronic pyelonephritis, despite the extensive pathologic changes and functional disturbances in the kidneys produced by all three species of bacteria, is not in keeping with the clinical impression that chronic pyelonephritis is a common primary cause of hypertensive vascular disease. It does, however, correlate with the autopsy studies of Shure which indicate that unless pyelonephritis is quite severe, the incidence of hypertension in individuals with chronic pyelonephritis is not greater than that in patients without this renal disease (10). It is thus of interest that a slight increase in blood pressure was found in the animals infected with *Proteus* since they sustained not only the most severe renal damage, but also occasionally suffered unequal damage to the two kidneys because of stones. Heptinstall and Gorrill, who studied the effect of bilateral and unilateral pyelonephritis in rabbits, only noted significant elevation of blood pressure in a few animals with unilateral nephrectomy and severe damage to the remaining kidney (11). It is conceivable, therefore, that with even more severe bilateral renal injury than observed in our experiments, or with a selectively unilateral injury, significant hypertension might develop. However, an alternative possibility for the clinical relationship between hypertension and chronic pyelonephritis is that impairment of renal function by chronic pyelonephritis renders the subject more susceptible to vascular stresses, including those of physical, dietary and behavioral origin, leading eventually to hypertensive vascular disease. This hypothesis is supported by the demonstration that hypertensive vascular disease occurring in rats with other types of renal injury than pyelonephritis can be intensified by steroids, by high sodium diets and by chronic behavioral disturbances (12–14).

Another probable explanation for the clinical relationship between chronic pyelonephritis and hypertensive vascular disease is that the kidney in hypertension, like the kidney in diabetes (15), is more susceptible to infection. Indeed, the frequent occurrence of pyelonephritis in malignant hyper-

FIG. 2C. CROSS SECTION OF KIDNEY INFECTED WITH *S. ZYMOGENES* (× 15)

Note marked pelvic thickening, dilatation of collecting tubules and infiltrative lesions in medulla, while the cortex is quite free of damage.

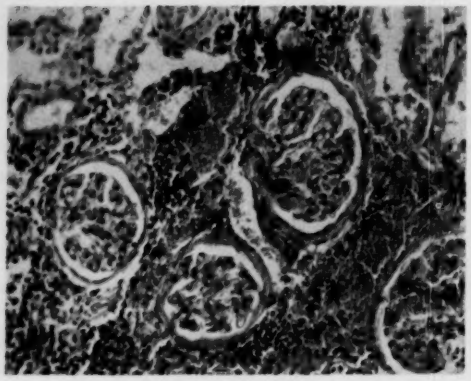

FIG. 3. SECTION THROUGH CORTEX OF KIDNEY INFECTED WITH *E. COLI* (× 280)

Note periglomerular fibrosis and otherwise intact glomeruli. Interstitial infiltration of mononuclear cells is present.

tension may represent, as Goldblatt contends (16), the imposition of an infection which then aggravates the hypertensive process. These problems are being investigated in our laboratory and results indicate that desoxycorticosterone acetate hypertension in rats increases susceptibility to acute pyelonephritis induced by *E. coli* (17) and that this infection at least temporarily aggravates the hypertension (18).

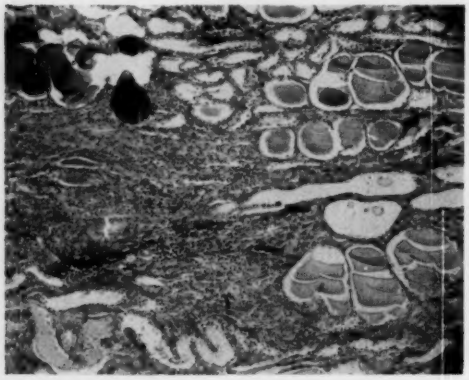

FIG. 4. SECTION THROUGH MEDULLA OF KIDNEY INFECTED WITH *E. COLI* (× 140)

Note tubular distortion and dilatation, colloid casts ("thyroid kidney") and interstitial infiltration and fibrosis.

SUMMARY AND CONCLUSIONS

Chronic pyelonephritis was produced in more than 200 rats by combining repeated intracardiac inoculation of either *E. coli, P. morganii* or *S. zymogenes* with renal massage. The animals were observed for as long as one year after initial infection. Since surgical intervention was not necessary to establish pyelonephritis, the resulting pathological lesions of the kidney were purely the product of chronic infection. Although a slight elevation of blood pressure above normal was noted in the rats infected by *Proteus*, significant hypertension did not develop. All groups demonstrated impaired renal function, as measured either by urea retention or by decrease in osmolal

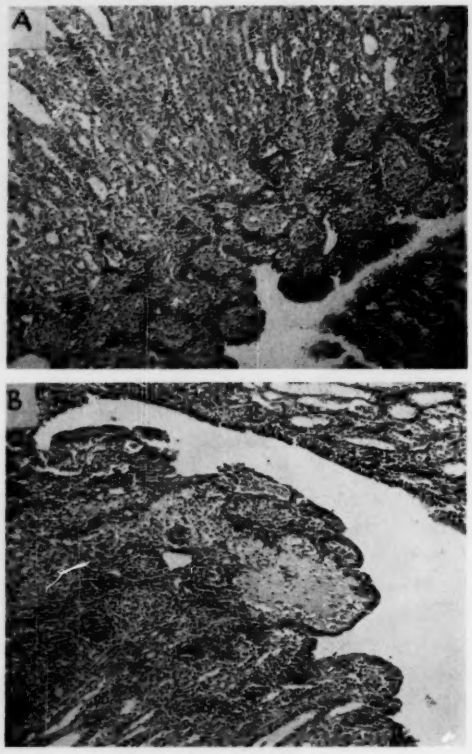

FIG. 5. SECTION THROUGH PELVIS OF KIDNEY INFECTED WITH *S. ZYMOGENES* (× 140)

Note "invagination" of pelvic mucosa into papilla in A. A "polypoid mass" in the pelvis consisting of pelvic mucosa and granulation tissue is shown in B.

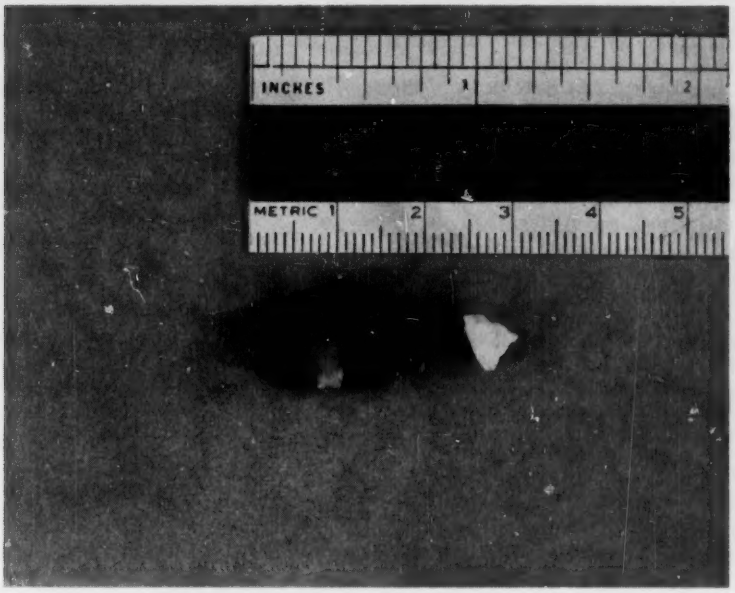

FIG. 6. CALCULUS WHICH FILLED ENTIRE PELVIS OF KIDNEY WITH CHRONIC INFECTION BY *P. MORGANII*

A sagittal section of a normal kidney is shown to illustrate size. Stone was composed of $MgNH_4PO_4$ (analysis by Dr. E. Prien, Boston, Mass.).

concentration of the urine after dehydration. Although the overall pathologic changes produced by all three organisms were basically similar and showed a striking resemblance to chronic pyelonephritis in man, certain features characteristic of each bacterial species were noted. Thus infection with *E. coli* was self-limited, but the inflammatory process that persisted in these sterile kidneys was at least as severe as that in rats with heavily persistent enterococcal infections of the kidney. *P. morganii* produced marked renal destruction, stone formation and hydronephrosis. Infection with *S. zymogenes* was still present in every rat examined at the end of one year, but observable lesions often predominated in the renal medulla.

The results demonstrate the importance of the bacterial species in determining the type and extent of renal injury in chronic pyelonephritis. Moreover, the absence of hypertensive vascular disease suggests several alternative hypotheses to the supposed etiologic relationship of hypertension to chronic pyelonephritis.

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THE EFFECT OF PROTEIN DEPLETION ON THE DISTRIBUTION OF PROTEIN SYNTHESIS IN THE DOG

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A well nourished adult dog can be maintained in nitrogen balance on a synthetic diet which provides 250 mg. of nitrogen as casein and 80 calories per Kg. body weight per day (1). If the protein in this diet is replaced isocalorically with carbohydrate, the dog continues to lose nitrogen in the urine, although at a rate which becomes progressively less. By the sixth week of protein free diet the urinary nitrogen has fallen to about 60 mg. per Kg. per day, which appears to be an irreducible minimum. If the animal is still kept on a protein free diet it becomes weak, apathetic, anorexic and edematous; unless it is given protein within about a week of the onset of these signs it dies. If the refeeding of protein is delayed too long the dog may still die although it is retaining nitrogen, or it may recover after a "lag" period. This observation has led to the hypothesis that severe protein depletion may eventually damage the protein synthetic mechanisms and thus lead to an irreversible state of depletion (2, 3).

In many tropical countries an analogous state is found in human infants. When they are taken from the breast they are fed a diet relatively adequate in calories but very poor in protein and as a result develop the clinical syndrome of kwashiorkor (4–6). A number of these children, on admission to hospital, fail to respond to protein and electrolyte repletion and die. The mortality rate in this unit is 20 per cent and the cause of death is not clearly defined. Other cases may show a "lag" period before they respond satisfactorily to treatment (6).

The work reported in this paper is an attempt to produce protein depletion of known severity in dogs and to correlate with the degree of depletion some change in protein metabolism detectable by an isotopic tracer technique. This technique was designed to be used on human cases and it was hoped that the results obtained under controlled conditions in dogs would provide a background for interpreting data obtained under much less precise conditions in children.

The methods used do not give a measure of the total amount of protein synthesized in unit time, but the results suggest that the distribution of synthesis between the various protein pools is altered by depletion. Thus, while the depleted animal may turn over a smaller amount of protein per day than the normal dog, a greater *proportion* of synthesis occurs in the active protein pools, such as plasma protein.

METHODS

Eight clinically normal adult dogs were fed a synthetic diet which provided 250 mg. nitrogen (as casein) and 80 calories per Kg. body weight per day (1). These dogs were in nitrogen equilibrium and are termed "normal dogs." Each normal dog was injected intravenously with 4 mg. T-1824 and approximately 4×10^6 cpm in 0.5 mg. S^{35} methionine per Kg. body weight. Four dogs received labeled DL-methionine and four received labeled L-methionine. In each group of four, two were started on a protein free diet on the day of injection and two were started on this diet one week after injection. The protein free diet was obtained by substituting sucrose isocalorically for casein.

The dogs were kept on the protein free diet and reinjected with T-1824 and S^{35}-labeled methionine after intervals of three to 10 weeks; four dogs were injected a third time after a similar interval. In some cases depletion was accelerated by bleeding, but at least a week was allowed between the last bleeding and the time of injection to enable the protein pools to re-equilibrate.

Daily collections were made of urine for 21 days; the feces for each one week period were pooled and the nitrogen content of the collections determined. When depletion was hastened by bleeding, the N content of the blood removed was also measured. It was therefore possible to follow the cumulative loss of nitrogen on the protein free diet; this is also expressed in Table I as the percentage loss of the initial body nitrogen, on the assumption that the normal dog contains 25.6 Gm. N per Kg. body weight.

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TABLE I

Change in body weight and nitrogen content of dogs fed a protein free diet

Expt. no.	Dog no.	Initial body wt.	Days on P.F.D.*	Body wt. at time of injection	Nitrogen lost during period				Initial† total body N	Total N lost	Body wt. lost
					Urine	Feces	Blood	Total			
		Kg.		*Kg.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	%	%
9	178	4.95	22	4.6	13.2	2.9	0	16.1	127	7.9	7.0
17	178	4.95	57	4.3	25.8	6.4	7.1	39.3	127	31.0	13.2
10	201	5.9	22	5.6	13.0	3.9	0	16.9	151	8.9	5.1
19	201	5.9	61	5.1	27.6	8.9	6.8	43.3	151	28.7	13.6
15	206	11.7	28	11.0	30.8	11.2	20.7	62.7	300	20.9	6.2
11	179	5.2	29	4.6	16.3	4.0	0	20.3	133	15.5	11.5
18	179	5.2	64	4.5	25.6	8.1	5.6	39.3	133	29.6	13.4
12	203	5.5	29	4.6	19.7	5.3	0	25.0	141	17.7	16.4
20	203	5.5	68	4.5	34.3	10.3	8.6	53.2	141	37.7	18.2
16	207	10.2	35	9.2	28.7	8.1	18.2	55.0	261	21.1	9.7
13	175	6.8	45	6.2	25.8	11.2	0	37.0	167	22.1	8.9
14	212	6.2	52	5.7	26.9	10.4	0	37.3	159	23.4	8.1

* P.F.D. = protein free diet.
† It is assumed that the body initially contained 25.6 Gm. nitrogen per Kg.

Venous blood samples were taken into heparin at one-quarter, one, two, four, six and eight hours after injection and then daily for one week. Plasma volume was measured in the usual way, by extrapolation to zero time from the dye concentrations in samples taken one-quarter and one hour after injection. Total plasma protein concentration was calculated from the N content of the samples taken just before each injection. Protein was taken as $N \times 6.25$ without allowance for nonprotein nitrogen.

For radioactivity measurements, plasma proteins were precipitated with 5 per cent trichloracetic acid (TCA). The activity of the supernatant was measured in samples taken up to eight hours after injection; these results are expressed as "cpm per ml. protein free plasma." In later samples the activity became so low that counts were not done on the supernatants.

The precipitated proteins were washed twice with 5 per cent TCA and then dissolved in N sodium hydroxide to give an approximately 5 per cent solution of protein. The activity in 0.25 ml. samples of this solution was measured. The N content was determined by micro-Kjeldahl and the results expressed as cpm per mg. of protein. These results represent the mixed proteins of plasma; no measurements were made on separated plasma protein fractions. Samples of the daily urine collections were counted but no counts were done on feces.

Radioactivity was measured with a gas flow counter, using 98.7 per cent helium and 1.3 per cent butane. Samples were prepared, dried and counted in disposable stainless steel cups. The results have been corrected for background, radioactive decay and self-absorption and are calculated to a standard dosage of 4.0×10^6 cpm per Kg. body weight. When second and third injections were made, the residual activity in samples taken immediately before reinjection was subtracted from subsequent values. This residual activity was less than 10 per cent of the dose administered and its rate of change less than 10 per cent over the time period of study; it has therefore been treated as a constant.

The term "specific activity" has been used in the text to mean radioactivity (from S^{35}) per mg. nitrogen. Strictly this is incorrect, but provided the sulphur/nitrogen ratio in the protein is constant the figure given is proportional to the true specific activity.

RESULTS

The extent of the depletion

Table I shows the estimated proportion of body N lost at various stages of depletion in relation to the loss of body weight. After 50 to 60 days on the protein free diet about 30 per cent of body N had been lost, whereas the weight fell by only 13 to 18 per cent. The discrepancy is presumably due to the accumulation of edema fluid (7), even though this could not be detected clinically, except in the very late stages. The distribution of the N loss between urine and feces is also shown in Table I.

Total circulating plasma protein

In four dogs successive measurements were made of plasma volume and plasma protein concentration, from which could be calculated the total amount of plasma protein in the intravascular compartment (Table II). It appears that in the later stages of depletion there is a rapid reduction in the amount of circulating protein, which outstrips the loss of total body N. This fits in with the clinical impression that in kwashiorkor the fall in plasma protein concentration is a late event in the development of the disease.

Disappearance of nonprotein-bound activity from plasma

The mean values and ranges for the samples taken up to eight hours after injection are shown in Figure 1. The curve was not significantly altered whether the dog was receiving protein or not, whether its protein stores were full or depleted, or whether DL- or L-methionine was given. The results have therefore been averaged.

Incorporation of labeled methionine into plasma protein

Figures 2 and 3 show the activity per mg. of plasma protein in samples taken one, two, four, six and eight hours after injection, in dogs given DL- and L-methionine. Again there is no apparent difference between the results with the DL- and L-forms.

The shape of the curve is similar in all experiments and reaches a maximum in about six hours. The height of the curve, however, was greatly increased in the depleted dogs (Table II, Column 9). In individual dogs the peak activity progressively rises with increasing depletion. Comparison between different dogs suggests that this process does not go on indefinitely, but may reach a maxi-

TABLE II

The effect of protein depletion on the size and activity of the intravascular plasma protein pool and on the apparent half-life of plasma protein in dogs injected with S^{35}-methionine

1	2	3	4	5	6	7	8	9	10	11
Dog no.	Days on P.F.D.*	Body N lost†	Plasma vol.	Plasma protein	Total plasma protein‡	Plasma protein lost‡	Isomer injected	Peak plasma protein activity	Total activity in plasma protein‡	Apparent plasma protein half-life
		%	*ml.*	*Gm./100 ml.*	*Gm.*	%		*cpm/mg.*	%	*days*
178	0	0	324	6.12	19.8	0	DL	41.5	4.15	13.6
178	22	7.9	310	5.81	18.0	9.2	DL	70.6	6.91	16.8
178	57	31.0	271	3.54	9.6	51.5	DL	120.7	7.37	12.8
201	0	0	346	7.04	24.4	0	DL	41.8	4.33	13.4
201	22	8.9	333	5.37	17.8	27.0	L	84.8	6.74	Not measured
201	61	28.7	343	3.54	12.2	50.0	L	112.2	6.71	16.0
179	0	0	339	6.10	20.7	0	DL	54.1	5.38	19.5
179	29	15.5	370	5.00	18.5	10.7	DL	73.4	7.40	36.0
179	64	29.6	292	3.38	9.9	52.1	DL	132.0	6.72	15.3
203	0	0	296	6.22	18.4	0	DL	50.4	4.22	9.0
203	29	17.7	363	4.64	16.8	8.8	L	84.3	7.70	Not measured
203	68	37.7	261	3.48	9.1	50.6	L	103.2	5.22	18.3
206	0	0		Not measured			L	56.0		13.8
206	28	20.9		Not measured			L	111.5		20.8
175	0	0		Not measured			L	46.5		16.6
175	45	22.1		Not measured			DL	97.7		Not measured
207	0	0		Not measured			L	56.7		16.4
207	35	21.1		Not measured			L	128.8		22.3
212	0	0		Not measured			L	65.7		13.7
212	52	23.4		Not measured			DL	133.3		Not measured

* P.F.D. = protein free diet.
† Refers to days on diet and N lost at the time of the injection of labeled methionine.
‡ Refers to plasma protein in intravascular compartment.

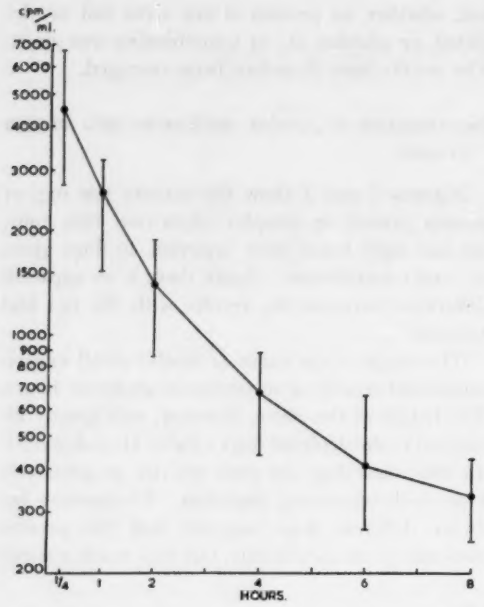

FIG. 1. DISAPPEARANCE OF RADIOACTIVITY FROM THE PROTEIN FREE FRACTION OF PLASMA: "FREE METHIONINE"

The curve is drawn through the mean values, and vertical bars indicate the range.

mum at about 20 per cent depletion of body N. In four dogs (Nos. 206, 175, 207 and 212, Table II), with an average depletion of 22 per cent of body N, the mean peak activity was 118 cpm per mg. plasma protein. In four other dogs (Nos. 178, 201, 179 and 203), with an average depletion of 32 per cent of body N, the mean peak was 117 cpm per mg. plasma protein.

Plasma protein decay curve

The apparent half-lives for the mixed plasma proteins are shown in Table II, Column 11. There is no consistent difference between normal and depleted dogs.

Excretion of labeled sulfur

Figures 4 and 5 show the daily excretion, expressed as per cent of the dose remaining in the body, in dogs given DL- and L-methionine. Again, there is little difference between normal and depleted animals. The excretion is perhaps a little more rapid in the depleted dogs, but the numbers are too few for the differences to be established with certainty. The specific activity (cpm per mg. N) of urine appears to reflect fairly closely that of plasma protein (Table III).

DISCUSSION

The striking difference between depleted and normal dogs is the increase in plasma protein specific activity as depletion progresses (Table II, Column 9). The higher specific activity can result from two factors: a reduction in size of the plasma protein pool, or a larger proportion of labeled molecules entering the pool. The first factor undoubtedly operates in depleted dogs; Table II, Column 7, shows that when 30 per cent of body N has been lost the circulating plasma protein falls to about half the normal amount. This, however, is not enough by itself to explain the increased specific activity of the plasma proteins, since Column 10 of Table II shows that in depleted dogs a larger *proportion* of the radioactivity was incorporated in plasma protein. The proportion is greatest when about 25 per cent of body N has been lost and thereafter appears to decrease again. Thus, if the proportion of dose incorporated in intravascular plasma protein in normal dogs is taken as unity, the four dogs in which measurements were repeated incorporated 1.60 times as much in moderate depletion and 1.44 times as much in severe depletion.

Yuile, Lucas, Neubecker and Whipple (8) reported that in dogs depleted by plasmapheresis as well as by a low protein diet the reduction in the extravascular extracellular protein pool was much greater than the reduction in circulating plasma protein. Their method of calculating the extravascular pool size is not given in detail. In the present experiments the size of this pool was not measured. However, it does not seem possible that a disproportionate reduction in the extravascular pool could account for the increased specific activity of the *intravascular* protein at six to eight hours after injection (Table II) since from the available data it probably takes at least two days for the two pools to reach equilibrium (9, 10).

Intravascular plasma protein is the only protein pool which can be quantitatively measured. The plasma volume, plasma protein concentration and

plasma protein specific activity can all be measured and hence the absolute quantity of radioactivity in intravascular protein can be calculated and expressed as a percentage of the administered dose. The result of this calculation is shown in Column 10 of Table II for the four dogs which were injected three times at varying stages of depletion. In the normal dogs an average of 4.5 per cent of the injected dose is accounted for by intravascular protein; the averages on the second and third in-

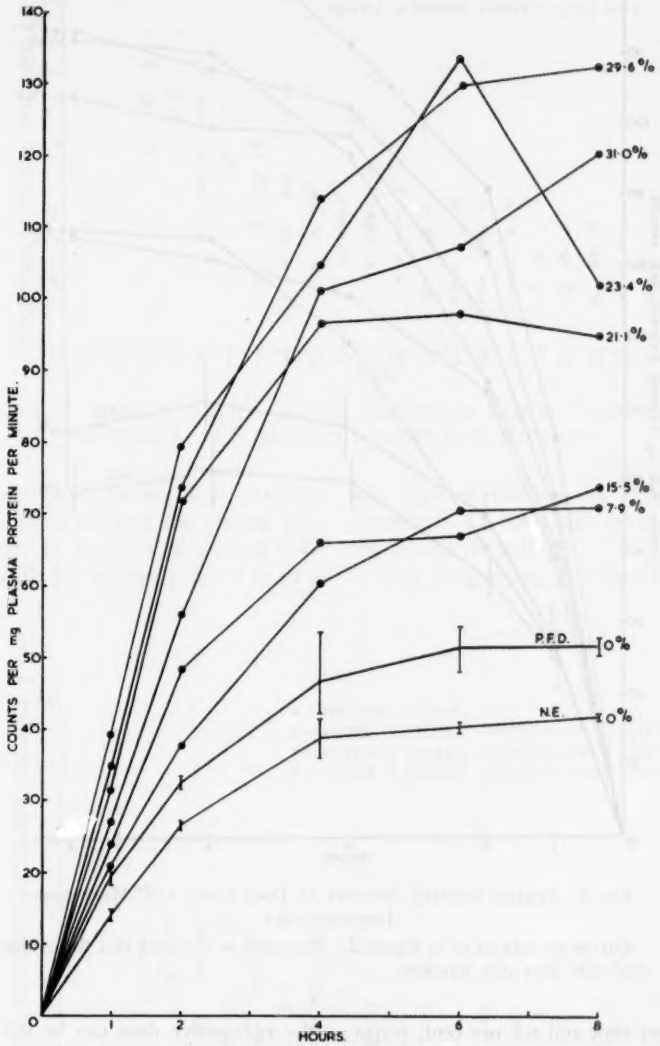

FIG. 2. PLASMA PROTEIN ACTIVITY IN DOGS GIVEN DL-S^{35}-METHIONINE INTRAVENOUSLY

Curve N.E. shows average values for two dogs in nitrogen equilibrium and Curve P.F.D. for two on protein free diet. The remaining curves are for dogs on protein free diet. The percentage of total body protein by which the dog had been depleted is shown to the right of each curve.

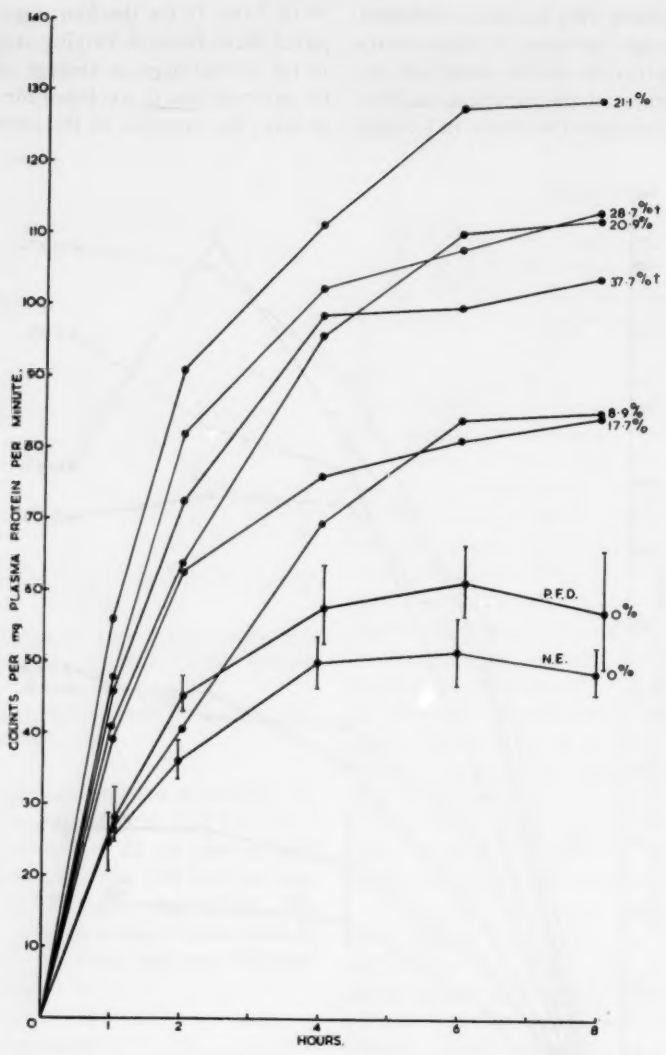

FIG. 3. PLASMA PROTEIN ACTIVITY IN DOGS GIVEN L-S^{35}-METHIONINE INTRAVENOUSLY

Curves are labeled as in Figure 2. The mark + indicates that these dogs died eight days after injection.

jections are 7.2 per cent and 6.5 per cent, respectively. It appears that with depletion an increasing proportion of the dose goes into intravascular protein, and that this effect is due to a change in the distribution of synthesis and not merely to an alteration in pool sizes, for the reason given above.

Something of the behavior of the remainder of the radioactive dose can be inferred from a consideration of the excretion of nitrogen and radioactivity in the urine and from the decrease in activity of the plasma protein. In the present experiments the values for apparent plasma protein half-life (Table II) are variable and show no consistent change with depletion. (The figures are

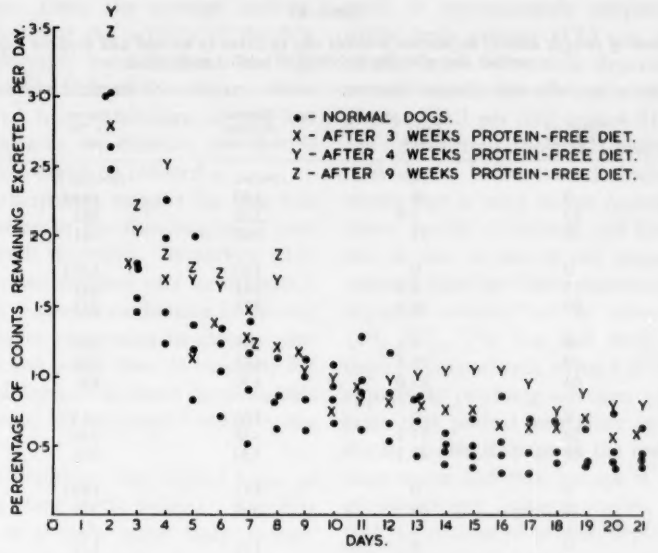
PERCENTAGE OF COUNTS REMAINING EXCRETED PER DAY.
DAYS.
• - NORMAL DOGS.
X - AFTER 3 WEEKS PROTEIN-FREE DIET.
Y - AFTER 4 WEEKS PROTEIN-FREE DIET.
Z - AFTER 9 WEEKS PROTEIN-FREE DIET.

FIG. 4. EFFECT OF DEPLETION ON THE EXCRETION OF LABELED SULFUR IN THE URINE AFTER INTRAVENOUS INJECTION OF DL-METHIONINE

not true half-lives owing to the reutilization of the radioactive label and to the fact that plasma proteins are a mixture of fractions of various half-lives.) Studies with I^{131}-labeled albumin in protein depleted children in Mexico (11) and in Jamaica (12) have also given no indication of any change in half-life.

With progressing depletion and a protein free

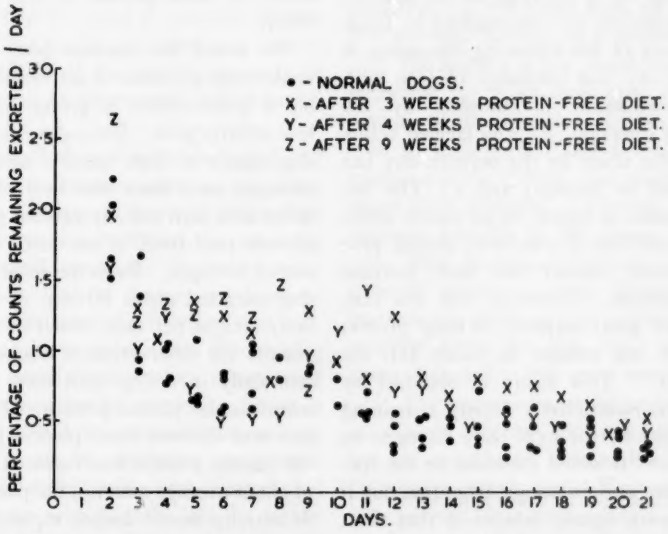
PERCENTAGE OF COUNTS REMAINING EXCRETED / DAY
DAYS.
• - NORMAL DOGS.
X - AFTER 3 WEEKS PROTEIN-FREE DIET.
Y - AFTER 4 WEEKS PROTEIN-FREE DIET.
Z - AFTER 9 WEEKS PROTEIN-FREE DIET.

FIG. 5. EFFECT OF DEPLETION ON THE EXCRETION OF LABELED SULFUR IN THE URINE AFTER INTRAVENOUS INJECTION OF L-METHIONINE

TABLE III

Comparison of specific activity in plasma proteins and in urine in normal and depleted dogs on the seventh day after the injection of labeled methionine

Dog no.	Days on P.F.D.	Body N lost	Plasma proteins on 7th day	Urine on 7th day	"Average" activity on 7th day*
		%	*cpm/mg. N*	*cpm/mg. N*	*cpm/mg. N*
178	0	0	140	144†	117
	22	7.9	342	354	128
	57	31	368	341	153
201	0	0	140	132†	118
	22	8.9	285	341	130
	66	28.7	375	355	146
179	0	0	195	279	123
	29	15.5	455	426	127
	64	29.6	450	453	150
203	0	0	156	143	122
	29	17.7	355	240	123
	68	37.7	430	305	159
206	0	0	175	189†	115
	28	20.9	390	371	142
207	0	0	172	157	122
	35	21.1	418	502	138

* Total radioactivity remaining on the seventh day (cpm) divided by total body nitrogen on seventh day (mg.).
† These dogs were receiving 0.25 Gm. casein N per Kg. per day and were in N equilibrium. The remaining dogs were on a protein free diet.

diet the excretion of nitrogen in the urine decreased, while the excretion of radioactivity tended slightly to increase (Figures 4 and 5).

Data concerning six of the dogs on the seventh day after their injections are summarized in Table III. For purposes of the following discussion it is assumed that: *a*) The exchange of free with protein-bound methionine is complete by the seventh day after injection; *b*) Any labeled sulfur not excreted in the urine by the seventh day has been incorporated in protein; and *c*) The behavior of methionine is typical of all amino acids.

The specific activities of urine and plasma protein are remarkably similar and both increase greatly with depletion. However, that this cannot be true of the great majority of body protein is shown by the last column in Table III, the "average activity." This figure is obtained by dividing the total radioactivity (cpm) remaining on the seventh day by the total body nitrogen on that day (mg.) and it would therefore be the specific activity of any protein sample from that dog if all the protein were equally labeled at that time. In the normal dog the urine, plasma protein and "average" activities are not greatly different (Table III). In the depleted dog the urine and plasma specific activities are two or three times greater than the "average," so there must necessarily be some protein of less than average activity.

No doubt the various body proteins have a continuous spectrum of activities from high to low, but it is convenient to group them into a high or low activity pool. Since the urine of the depleted dog shows a high specific activity the urinary nitrogen must have been derived from the breakdown of a high activity protein pool. The plasma protein pool itself is too small to supply the excreted nitrogen. Even the most severely depleted dog excreted about 80 mg. of nitrogen per Kg. body weight per day, which in a 5 Kg. dog represents the destruction of about 2.5 Gm. of protein daily in a dog with only about 10 Gm. of intravascular plasma protein. If the urinary nitrogen was derived from plasma protein alone and the plasma protein was replaced from a large pool of about average activity, the plasma protein specific activity would decline rapidly and this has not been found to be the case. It is therefore necessary to postulate a fairly large high activity pool

with two or three times the average activity. Clearly this implies that the activity of the low pool must be considerably lower than the "average activity" and relatively little of the urinary nitrogen can be derived from catabolism of this low activity pool; otherwise, by dilution, the specific activity of the urine would be reduced.

Some animal experiments support the idea that there is an alteration in the distribution of protein metabolism with depletion. Waterlow (13) has found that protein-depleted rats incorporate a greater proportion of labeled methionine in visceral protein and a smaller proportion in carcass protein (muscle, skin and bone) than do normally fed rats. A similar difference is found between rats having 15 per cent or 30 per cent of casein in the diet (14).

During protein depletion two related types of change are taking place in the animal. The first is the sacrifice of protein from body tissues. There is information from many sources that this affects different organs and tissues to different extents and that the distribution of depletion varies with the duration of protein lack. Liver protein bears the greater part of the loss at first (14–17). Some plasma protein is also sacrificed, the albumin more rapidly than the globulin fractions (7). When these "labile" stores are exhausted the loss is borne by the large mass of protein of slower turnover, which is used to maintain plasma protein (18). Changes of this kind are detectable by measurements of the protein contents of organs and represent an anatomical redistribution of protein.

The second type of change is physiological and is detectable only by tracer studies in the intact animal. Just as the site of protein destruction changes with progressive depletion, so does the distribution of protein synthesis. From the present results we may think of the protein-deprived animal drawing in its metabolic frontiers and concentrating its synthetic resources on a diminishing proportion of its protein mass, which presumably is the most important for its survival. There is some indication that this compensatory mechanism tends to break down as depletion progresses. The proportion of labeled amino acid incorporated into plasma protein was slightly less in severe than in moderate depletion. It is perhaps significant that the loss of plasma protein (13.9 per cent) is approximately proportional to that of whole body protein (12.5 per cent) in the first month or so of protein deprivation, but in the second month the plasma protein loss is much greater (51.1 per cent against 31.7 per cent; average figures from Table II, Column 6).

Whipple, Miller and Robscheit-Robbins have shown that in very severe depletion the raiding of tissue protein decreases, and have suggested that this is due to loss of cell enzymes (18). This enzyme loss has been demonstrated in protein-depleted animals and in malnourished children (19, 20). The fact that death may occur in a depleted animal even when it is being re-fed and is apparently retaining nitrogen (2, 3, 6, 18) indicates that protein loss alone is not the cause of death. Some damage to the metabolic machinery must occur and this damage is not detectable by gross nitrogen balance studies. It is suggested that the concept of protein depletion is incomplete if it is based only on the consideration of the state of the protein stores. The redistribution of protein metabolism, as revealed by tracer studies, may be physiologically a more important factor.

SUMMARY

1. $^{*}S^{35}$-labeled methionine was injected into normal and protein-depleted dogs. The degree of depletion was calculated from the cumulative negative nitrogen balance on a protein free diet.

2. The activity of the mixed plasma proteins was measured for eight days after injection and the activity of the urine for 21 days.

3. In both normal and depleted dogs the peak plasma protein activity was reached at about six hours after injection. Depletion did not alter the apparent half-life of the plasma proteins.

4. With increasing depletion, up to a loss of some 25 per cent of body N, there was a marked increase in peak specific activity of the plasma proteins (cpm per mg. N). With greater degrees of depletion this trend tended to be reversed.

5. The increased specific activity of the plasma proteins in depleted dogs can be explained partly, but not entirely, by a reduction in the size of the circulating protein pool. Even when this is taken into account, it is shown that a larger proportion of the injected dose is incorporated in plasma protein in the depleted than in the normal animal.

6. The reasons for this are discussed: It is suggested that the effect represents a concentration of protein synthesis in the more essential organs, at the expense of the less essential, such as muscle. In the most advanced stages of protein depletion this compensatory mechanism may break down.

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EFFECT OF CHOLESTEROL SYNTHESIS INHIBITION IN NORMOCHOLESTEREMIC YOUNG MEN *

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Recently the concept of using specific inhibitors of endogenous cholesterol synthesis as a means of reducing the cholesterol content of the body has been reviewed (1). One such specific inhibitor is the metal vanadium. Salts of vanadium have been shown to inhibit the utilization of mevalonic acid for cholesterol biosynthesis (2), to inhibit cholesterol biosynthesis *in vitro* and *in vivo* (3), to induce mobilization of predeposited aortic cholesterol in rabbits (4) and to lessen the deposition of cholesterol in rabbits (5) and in birds (6) fed high cholesterol diets.

To extend these studies to man, the effects of vanadium on sterol balance in normocholesterolemic men under rigid dietary control were examined. The investigations reported here show that, under such conditions, inhibition of endogenous cholesterol biosynthesis with a salt of vanadium produces a lowering of serum cholesterol levels. The studies provide data which suggest that mobilization of tissue cholesterol stores also occurs.

MATERIAL AND METHODS

A. Experimental group. The subjects for the study were five normal male medical students, 23 to 26 years of age. The diets were prepared in a separate kitchen and the students ate in a private dining room. During the period of the study a daily weight record of the men showed variations of less than 3 per cent.

B. Diet. The diet consisted of an identical daily intake of the same items divided into three meals plus a bedtime snack. The diet contained 109 Gm. of protein, 58 Gm. of fat and 321 Gm. of carbohydrate each day for a total of 2,242 calories. Two of the men, R. M. and E. W., received an additional 363 calories as bread and jelly to maintain their weight during the last three weeks of the study. All of the items in the diet with the exception of lettuce, tomatoes and bread were utilized from a single bulk lot purchased at the start of the experiment. Fat was supplied primarily from lean hamburger, butter, ice cream and bologna. Black coffee and tea were allowed as desired. One decavitamin tablet (U.S.P.) was given each day to obviate any possible change in available vitamins induced by modification of intestinal flora due to the vanadium. By careful weighing of portions and use of constant temperatures in cooking, variations in fat content were kept to a minimum. Weekly analyses of a total day's portion of food revealed variations of less than 2 per cent in fat and dietary sterol intake.

C. Experimental design. The men ate an identical diet during the entire study. After a two week control period, each man received diammonium oxy-tartratovanadate[1] (B. B., 100 mg.; others, 125 mg.) orally in three divided doses each day for the experimental period of six weeks. During the final recovery period of three weeks the vanadium administration was discontinued.

D. Chemical methods. Serum. Fasting blood samples were obtained once each week. Cholesterol (7), phospholipid (8, 9), triglyceride (10), alkaline phosphatase (11) and transaminase (12) serum levels were determined. Serum cholesterol determinations were carried out on duplicate aliquots of sera.

Urine. Twenty-four hour samples were collected once each week and daily for the week after cessation of vanadium administration. Vanadium was determined by a colorimetric method (13) as well as spectrographically.[2] 17-Ketosteroids and 17,21-dihydroxy-20-ketosteroids were determined at the start and end of the period of vanadium administration (14, 15). Routine urinalyses were performed on all collected urines.

Feces. The total fecal output was collected for a 72 hour period each week. The stools were immediately frozen, then combined, lyophilized and stored at 4° C. The total 72 hour sample was ground and stored *in vacuo* over P_2O_5 while analyses were in progress. Three weighed aliquots

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[1] Generously supplied by Dr. Frederick Heath of Merck, Sharp and Dohme.

[2] These determinations were performed by Mead Johnson Company through the kind offices of Dr. Thomas Fleming.

of each pooled 72 hour sample were extracted with acetone-ethanol (1:1) for 48 hours at room temperature for determination of total digitonin precipitable sterols (16), digitonin precipitable, Liebermann-Burchard (L.B.) positive sterols (7) and total 3α and β-OH L.B. positive sterols (17). Using this extraction procedure and triplicate samples of dried, powdered feces, these sterol procedures were found to give as good recoveries of sterol as is the case when serum is analyzed. Some difficulty was experienced with nonspecific chromagens with the Abell, Levy, Brodie and Kendall procedure (17). For this reason, with both procedures utilizing the L.B. reaction, a correction for "fast acting" sterols was made by a two minute sample (18). Most of the "fast acting" sterols are precipitated by digitonin and are accounted for in the digitonin precipitable, L.B. negative sterol fraction. Digitonin precipitable, L.B. negative sterols were calculated by subtracting the digitonin precipitable, L.B. positive sterols (7) from the total digitonin precipitable sterols (16). Other weighed aliquots of feces were extracted with ethanol in a Soxhlet extractor for 24 hours and the extract analyzed for bile acid content (19). Total fat content was also determined on aliquots of the dried feces (20).

Diet. Aliquots of the total 24 hour diet, prepared by homogenization in a Waring Blendor with water, were analyzed for total fat (20) and neutral sterol (7, 16, 17) content.

E. Statistics. A statistically significant difference in this paper is defined as a probability of <0.05 as calculated from the expression:

$$\text{"t"} = \frac{\text{difference of the means}}{\text{standard error of the difference of the means}},$$

where

$$\text{standard error of the difference of the means} = \sqrt{\frac{\Sigma d^2}{N(N-1)}}.$$

F. Calculations. From Figure 1 it is seen that: 1) $I = -740 + {}^{A}D$ + gut sterol output; 2) $I = {}^{AF}D$ + E.S. + B.A.; where ${}^{A}D$ = dietary sterol absorbed, calculated by method of Cook, Edwards and Riddell (21) (see Discussion); ${}^{AF}D$ = amount of ${}^{A}D$ excreted in 24 hours = 0.25 ${}^{A}D$ (22); 740 = total sterol in diet; BA = bile acids; ES = endogenous neutral sterols and I = total body sterol output to gut.

The total endogenous sterol output from the body per 24 hours (E.S. + B.A.) must equal the synthetic input per 24 hours plus any previously existent sterol excreted during that 24 hours; 3) E.S. + B.A. = S + ΔB, where S = endogenous sterol synthesis, ΔB = change in body sterol content = body sterol content in vanadium period—body sterol content in recovery period = $B_V - B_R$ (V = vanadium period, R = recovery period). Substituting Equation 3 in 2: 4) $I = {}^{AF}D + S + \Delta B$.

Again referring to Figure 1, it is seen that a change in the body sterol must represent the sum of the changes in the serum and tissue sterols: 5) $\Delta B = \Delta Se + \Delta T$, where ΔT = change in tissue sterol content = tissue sterol content in vanadium period—tissue sterol content in recovery period = $T_V - T_R$.

ΔSe = change in total serum cholesterol = total serum cholesterol content in vanadium period—total serum cholesterol content in recovery period = $Se_V - Se_R$.

From the above equations, ${}^{A}D_R$, ${}^{A}D_V$, ${}^{AF}D_R$, ${}^{AF}D_V$, I_R, I_V, ΔB_V and ΔT_V are calculated as shown in Addendum.

S_R is obtained from Equation 4 by assuming that the steady state has again been reached and ΔB_R is zero (see Discussion).

S_V is obtained by assuming that the ratio I_V/I_R is proportional to the ratio S_V/S_R (see Discussion).

All quantities are expressed as mg. of cholesterol per day.

RESULTS

Serum lipids

The results of serum determinations for total cholesterol, free cholesterol, phospholipid and triglyceride are given in Table I. In Figure 2 the means of the weekly cholesterol determinations

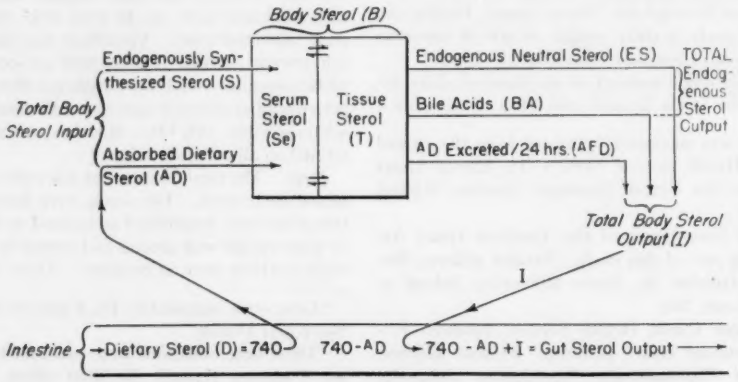

FIG. 1. SCHEMATIC PRESENTATION OF BODY-INTESTINE STEROL INTERCHANGE

TABLE I

Serum lipids and fecal sterol excretion

Subject	Week*	Body wt.	Serum lipids: Total cholesterol	Serum lipids: Free cholesterol	Serum lipids: Phospholipid phosphorus	Serum lipids: Triglyceride	Serum lipids: C/P ratio	Fecal lipids and fecal bile acids: Dry wt.	Fecal lipids and fecal bile acids: Fat†	Fecal lipids and fecal bile acids: L.B. positive sterols‡	Fecal lipids and fecal bile acids: Digitonin precipitable L.B. negative sterols	Fecal lipids and fecal bile acids: Bile acids
		lbs.	*mg. %*	*mg. %*	*mg. %*	*mg. %*		*Gm./24 hrs.*	*Gm./24 hrs.*	*mg./24 hrs.*	*mg./24 hrs.§*	*mg./24 hrs.*
B. B.	C2	149	150	37	8.62	147	17.4	30.7	3.6	676	152	530
	V2	148	149	33	8.50		17.5	30.2		565	79	412
	V4	146	146	34	8.67		16.9	29.6		778	36	464
	V6	147	138	34	9.30	194	14.9	27.1	3.5	510	40	472
	R3	146	141	34	8.55	172	16.5	28.2	3.2	606	56	499
W. B.	C2	154	149	31	8.42	135	17.7	29.1	3.8	597	244	247
	V2	154	141	28	7.95		17.8	27.5		497	109	176
	V4	153	145	34	8.45		17.2	25.3		367	122	118
	V6	152	119	24	8.25	183	14.4	22.2	3.0	328	5	176
	R3	151	139	30	8.55	168	16.3	26.3	4.0	450	169	311
R. M.	C2	175	234	63	11.00	168	21.3	30.6	3.5	470	113	252
	V2	175	235	62	10.52		22.4	33.9		731	91	192
	V4	174	229	58	10.90		21.0	28.7		704	100	198
	V6	173	190	49	10.60	233	17.9	30.4	3.3	435	71	264
	R3	172	219	54	10.10	158	21.7	28.6	3.2	515	59	266
D. S.	C2	151	164	39	9.00	140	18.2	27.3	2.6	482	167	365
	V2	150	173	43	9.22		18.8	23.8		411	81	316
	V4	150	179	44	9.32		19.2	20.3		344	59	222
	V6	150	138	31	8.80	227	15.7	14.7	2.8	236	12	185
	R3	150	157	36	9.05	190	17.4	17.4	3.0	294	113	287
E. W.	C2	170	167	44	8.08	250	20.7	29.6	4.1	589	146	570
	V2	168	181	44	9.22		19.7	28.6		581	125	401
	V4	167	179	44	9.57		18.7	29.5		542	9	725
	V6	166	168	38	9.85	246	17.1	32.6	3.9	497	60	468
	R3	167	187	41	9.37	214	20.0	28.7	3.8	508	150	705

* C = control; V = vanadium; R = recovery. Serum values are those of last day of the respective week.
† Diet contained 58 Gm. of fat per 24 hours.
‡ Diet contained 531 mg. L.B. positive sterols per 24 hours.
§ Diet contained 209 mg. digitonin precipitable, L.B. negative sterols per 24 hours.

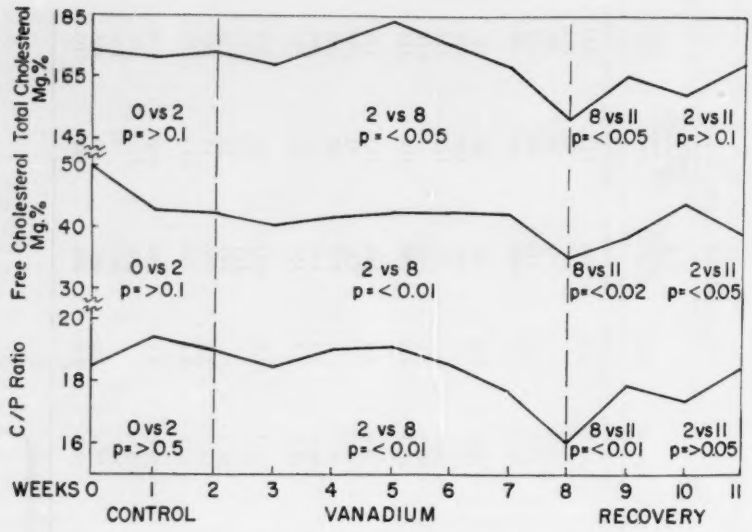

FIG. 2. CHANGES IN MEAN SERUM LIPIDS IN NORMOCHOLESTEREMIC MALES RECEIVING DIAMMONIUM OXY-TARTRATOVANADATE

Probability that given means differ significantly from each other is shown with weeks compared beneath each curve.

and the cholesterol/phospholipid phosphorus (C/P) ratio are graphed. The total cholesterol and the free cholesterol levels of the serum fell by a statistically significant amount during the six weeks of vanadium administration. The statistically more significant fall in the free serum cholesterol might be anticipated since the free serum cholesterol is a more direct indication of the rate of hepatic synthesis (23). Three weeks after cessation of vanadium administration both the total and free serum cholesterol showed a statistically significant elevation from their reduced values at Week 8.

The serum phospholipids did not show any statistically significant trend after vanadium administration and the C/P ratio showed a very significant decline by Week 8 and a significant increase over the Week 8 value by the end of the recovery period. If, as some investigators believe (24), the C/P ratio is the most reliable indication of the atherogenicity of the serum lipids, then cholesterol synthesis inhibition has, in a period of six weeks, produced a beneficial change in this index.

It can be seen in Table I that the serum triglycerides rose during the period of vanadium administration. On statistical analysis this rise is found to be significant at $p < 0.05$ and the consequent lowering after cessation of vanadium also to be significant at $p < 0.05$. Whether the change in triglycerides is due to some direct action of vanadium, such as increased synthesis of triglyceride secondary to inhibition of phospholipid synthesis (25), or to a mobilization of fat resultant from the lowering of serum or liver cholesterol levels is not known at this time.

Fecal sterols and bile acids

In Table I the results of determinations done on the feces are tabulated. From the lack of change in the fat content (Column 10) it is clear that vanadium did not interfere with fat absorption from the gastrointestinal tract. In Figure 3 the mean 24 hour fecal content of the various sterol fractions and the bile acids are graphed separately and as total sterol excretion. The top curve shows the decline in total fecal sterol excretion ($p < 0.05$) during the period of vanadium administration and then the rise ($p < 0.05$) during the recovery phase after vanadium. The total L. B. positive sterols as determined by the

Abell, Levy, Brodie and Kendall procedure (17) and the digitonin precipitable, L. B. negative sterols declined significantly during vanadium dosage but the decrease in the bile acids was not significant when considered separately.

Relation of absorbed vanadium to effects on sterol balance

It is known that urinary vanadium represents approximately 60 per cent of the vanadium absorbed from the gastrointestinal tract during the previous 24 hours (26). Urinary vanadium levels as determined weekly by a dye method (13) showed a wide variation both from one week to the next for the same individual and from one subject to another on the same dosage of diammonium oxy-tartratovanadate. These values ranged from 4 to 100 μg. V/L. urine. It soon became obvious that the absorption of vanadium from the gastrointestinal tract was most variable. However, at the conclusion of the vanadium administration period, total urine was collected for the next five days and analyzed for vanadium content by a chemical and a spectrographic method. This vanadium represented that which had been absorbed and retained in the body. In Figure 4 the residual vanadium has been plotted against the per cent reduction in the serum free cholesterol for each of the men in the study. It is clear that a relationship exists between the retained vanadium and the lowering of the serum free cholesterol. In Figure 5, using the spectrographic data, we find a similar curve which suggests that a concentration of approximately 1 μg. vanadium per pound body weight is necessary for a maximal effect.

Vanadium toxicity

There were no overt evidences of toxicity from vanadium in any of the men. Complete blood counts including platelets, routine urinalyses, blood urea nitrogen and blood glucose remained unchanged during the six weeks of vanadium administration. Liver functions as determined by serum cholesterol esters, serum alkaline phosphatase, serum transaminase and serum bilirubin were unchanged. The possibility existed that inhibition of cholesterol synthesis might also

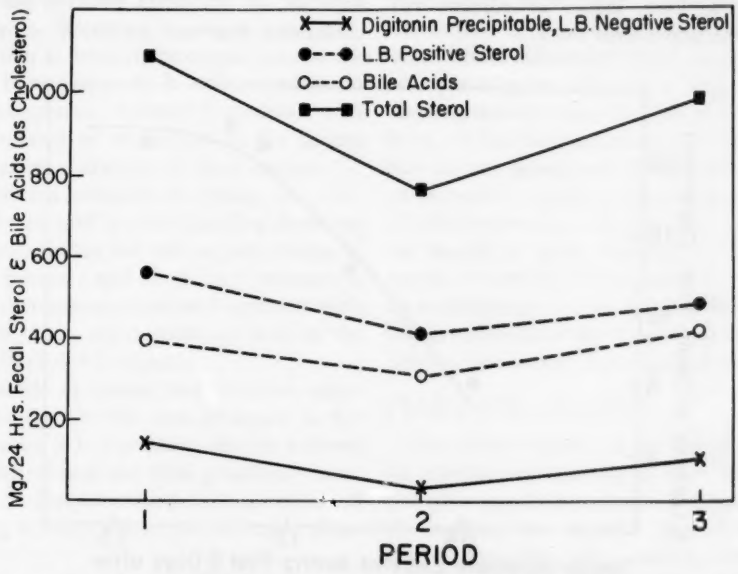

FIG. 3. CHANGES IN MEAN FECAL STEROL AND BILE ACID EXCRETION INDUCED BY INHIBITION OF ENDOGENOUS CHOLESTEROL SYNTHESIS

1 = end of control period; 2 = end of vanadium period; 3 = end of recovery period.

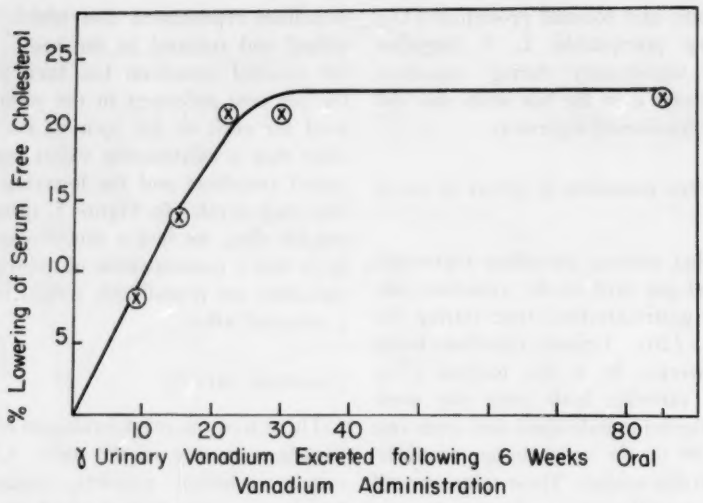

FIG. 4. RELATIONSHIP BETWEEN FALL IN SERUM FREE CHOLESTEROL AND RETAINED VANADIUM

inhibit adrenal corticoid production, but 24 hour urinary 17-ketosteroid and 17,21-dihydroxy-20-ketosteroid excretions were not lowered by the six weeks of vanadium administration. Thus no evidence of measurable toxic effect of vanadium on bone marrow, liver, kidney or adrenal was found during this short-term study.

DISCUSSION

From the data presented in this paper, the reader may have derived the following impressions: *1*) The reduction in serum cholesterol is minimal; *2*) no direct evidence for endogenous cholesterol synthesis inhibition is provided; and

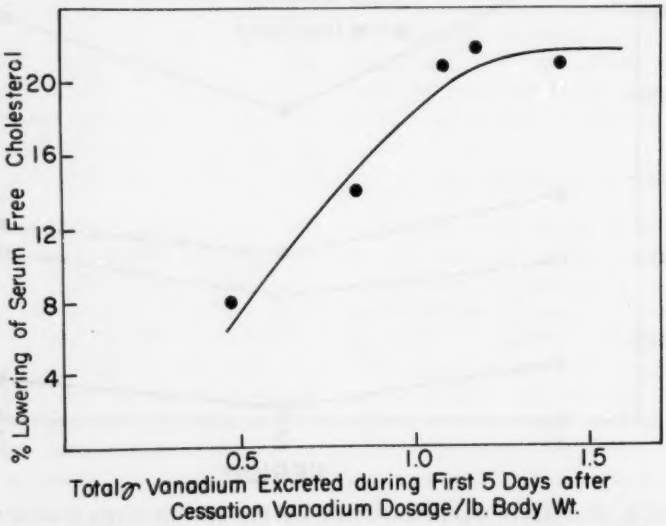

FIG. 5. RELATIONSHIP BETWEEN LOWERING OF SERUM FREE CHOLESTEROL AND RETAINED VANADIUM PER POUND OF BODY WEIGHT

3) the reduction in fecal sterol output is subject to a variety of interpretations.

In discussing the first of these points, it is clear that the reduction in serum cholesterol after six weeks of vanadium administration is small; the maximal reduction in free cholesterol is about 20 per cent. However, it must be noted that the subjects of this study were normocholesteremic young men with presumably normal rates of cholesterol synthesis and with normal plasma lipoprotein transport systems. Under such conditions it seems unwarranted to expect a marked change in serum cholesterol after a short period of inhibition of endogenous cholesterol synthesis.

The second point, concerning evidence for cholesterol synthesis inhibition, must be approached indirectly if one is to avoid the use of radioactive tracers in normal humans. Vanadium has been shown to inhibit cholesterol synthesis in animals by direct means (3, 4). To qualify as potentially effective in man, an inhibitor of cholesterol synthesis must be shown to block one of the steps between acetoacetate and squalene in the synthetic sequence of cholesterol and further, the inhibition of human tissue cholesterol synthesis must be demonstrated (1). Vanadium as diammonium oxy-tartratovanadate, has been reported to fulfill these criteria (*2, 27*).

The reduction in fecal sterol output may be interpreted in three ways: *1*) It may represent no change in endogenous cholesterol synthesis with an actual retention of cholesterol in the tissues and an equivalent reduction in fecal output; *2*) it may represent a reduction in endogenous cholesterol synthesis with a corresponding lowering of fecal sterol excretion but with no net change in tissue sterol content; and *3*) it may represent a reduction in endogenous cholesterol synthesis with a depletion of tissue sterol stores as well as the reduction in fecal sterol output.

It is impossible to choose between these alternatives on the basis of the data presented in the paper. However, it is possible to furnish indirect evidence which makes the first possibility most unlikely and to suggest a mathematical means of differentiating between the second and third alternatives.

Let us first consider the possibility that tissue sterol stores have actually increased during the period of vanadium administration with no change in cholesterol synthesis. The evidence that cholesterol synthesis is inhibited has been given. Mountain, Stockell and Stokinger found lowered tissue sterol stores after vanadium administration in normocholesteremic rabbits (5). Such direct data for man are not available. However, suggestive evidence is provided by cholesterol analyses of livers from two patients with brain tumors who had received diammonium oxy-tartratovanadate for four and eight months, respectively. The liver cholesterol levels were 225 and 250 mg. per 100 Gm. of liver, values below the average normal of 300 mg. per 100 Gm. for a 70 Kg. man (28).

To enable us to choose between the possibilities that tissue sterol stores have remained the same or decreased, we must discuss the validity of our balance methods and the procedure for calculating the tissue sterol stores from the balance data.

In order to evaluate the effect of a cholesterol synthesis inhibitor on sterol balance in man under rigid dietary control, it is necessary to measure the dietary sterol, the fecal neutral sterol and the fecal bile acids. Then, assuming the amounts of sterols lost by excretion in urine to be insignificant and those lost in sebum to be constant and knowing in this case that conversion to steroid hormones is unchanged, the sterol balance may be determined by the calculations given in the Methods. These calculations were devised as a means of separating the effect of a drug on the serum cholesterol level from its effect on the tissue sterol level. It has been apparent to clinicians for years that in any given patient the level of the serum cholesterol is a poor means of judging the degree of atherosclerosis. By the same token, perhaps we should be more reserved in using a fall in serum cholesterol after a particular type of therapy as an indication of a beneficial action. By analogy one is reminded of the frequent lack of correlation between serum and tissue potassium levels.

Validity of the calculations

The initial validity of the calculations depends on whether the lowering of total fecal sterol output after vanadium is due to increased destruction of sterol by the bacterial flora of the intestinal tract. To solve this problem, feces with and without added vanadium were incubated and then analyzed. No change in total fecal sterol content was found.

TABLE II

Calculated changes in body and tissue sterol content during vanadium administration*

Subject	Recovery period, S_R	Week of vanadium dosage: 2 Sv	2 ΔSe	2 ΔB	2 ΔT	4 Sv	4 ΔSe	4 ΔB	4 ΔT	6 Sv	6 ΔSe	6 ΔB	6 ΔT
B. B.	545	465	+ 7	−11	−18	513	+ 5	+35	+30	520	− 3	−30	−27
W. B.	356	239	+ 3	−21	−24	189	+ 8	−57	−65	240	−31	−90	−59
R. M.	107	34	+16	+88	+72	40	+14	+59	+45	105	−52	−15	+37
D. S.	135	155	+11	+31	+20	90	+21	−21	−42	65	−31	−71	−40
E. W.	645	391	− 4	− 2	+ 2	711	− 9	−27	−18	457	−36	−39	− 3

* Expressed as mg. cholesterol per day; S = synthesis; ΔSe = change in total serum cholesterol content; ΔB = change in total body sterol content; and ΔT = change in total tissue sterol content.

Without the use of isotopes it was, of course, impossible to assay directly the amount of absorbed dietary sterol. By the method of Cook, Edwards and Riddell (21) the increment in sterol absorption over that existing in the control period is measured. Thus our calculated amounts of $^{A}D_R$ and $^{A}D_V$ are each too small by the quantity of dietary sterol absorbed in the control period. However, as can be seen from the example in the Addendum, even if this missing quantity were as high as 400 mg. (representing an unlikely 60 per cent absorption in the control period) the change in the I_V/I_R ratio would be only 2 per cent. Also, any increase in $^{AF}D_V$ would increase the significance of the negative change in ΔB.

To calculate the synthesis during the recovery period, it is necessary to assume that the steady state has again been reached and that ΔB_R is zero. Because it is likely that, in the absence of a lowering of cholesterol endogenous synthesis, man is always storing some cholesterol, in actuality ΔB_R is probably a very small positive number. However, this constant storage of sterol must be so small on a daily basis that our assumption that it is zero should not influence the far larger changes found in sterol output.

Finally, to calculate the synthesis in the vanadium periods, it is necessary to assume that the ratio I_V/I_R is proportional to the ratio S_V/S_R. This assumption is justified because S is the major component of I and because the reduction in synthesis after vanadium so calculated is always less than might be expected from direct isotopic measurements of synthesis depression in animal and human tissues in *in vivo* experiments (4, 27) under similar conditions of vanadium dosage.

It is likely, therefore, that these calculations express the minimal reduction in tissue sterol content which has occurred, because the period of greatest change (*i.e.*, from the control to the vanadium periods) is not used and because the calculated rate of synthesis inhibition is minimal. It must be stressed that, due to the nature of the assumptions relating to the steady state during and after vanadium, the values calculated for the various entities cannot be regarded as absolute amounts. Further, it is clear that ^{A}D and ^{AF}D can be more exactly estimated by methods more elegant than those available for this study. However, in the opinion of the authors, the logical basis of the calculations is sound and accordingly the trend shown by ΔB and ΔT is correct.

Despite the factors which tend to minimize the calculated effects of cholesterol synthesis inhibition, it can be seen from Table II that in all the five men receiving vanadium for six weeks a reduction in body sterol content (ΔB_V) occurred. The importance of the concept of calculating the change in tissue sterol content (ΔT) becomes clear when one notes that all the men showed a reduction in total serum cholesterol (ΔSe) whereas only four men actually were lowering their tissue sterol stores (ΔT). For example, if one judged the results of vanadium on the basis of lowering of serum cholesterol alone he would think that R. M. showed the greatest effect, while in actuality R. M. was still depositing sterol in his tissues.

The trend of ΔB and ΔT during the period of vanadium administration shows the progressive effect of cholesterol synthesis inhibition. The mean figures for the five men are presented graphically in Figure 6. Both ΔB and ΔT have remained positive until Week 2. By Week 6 both ΔB and ΔT have become negative. It should

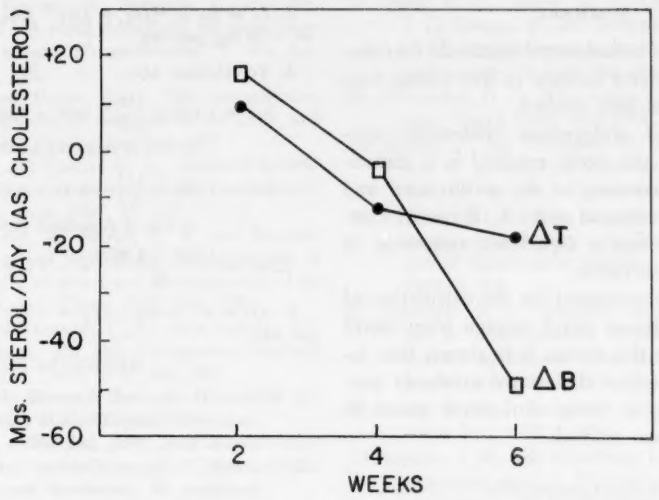

FIG. 6. TREND OF MEAN ΔB AND MEAN ΔT DURING VANADIUM ADMINISTRATION

ΔB = body sterol content in given week of vanadium period minus body sterol content in recovery period; ΔT = tissue sterol content in given week of vanadium period minus tissue sterol content in recovery period.

be noted again that the fall in ΔB is more dramatic, emphasizing the influence of the relatively labile serum cholesterol. However, the lesser decrease in ΔT is progressive and the decline from Week 2 to Week 6 is statistically significant at the < 0.05 level.

London and Rittenberg, using D_2O, calculated the amount of serum cholesterol synthesized per day by a normal young man to be 546 mg. (29). More recently, Taylor, Cox, Davis and Cross have measured directly the synthesis of cholesterol from acetate in several hundred human livers (30). Our mean of 450 mg. of cholesterol synthesized per 24 hours [this quantity includes measured urinary corticoids and an estimated skin sterol synthesis per 24 hours of 75 mg. (28) at the end of the recovery period (Table II)] is roughly four times their mean obtained on patients undergoing surgery. However, allowing for the nutritional differences involved and the fact that they measure only hepatic cholesterol synthesis, our calculated amounts of total body synthesis tend to support Taylor and co-workers' contention that the true human cholesterol synthesis rate may be far lower than currently accepted estimates.

Throughout this study it has been assumed that the effects measured were attributable to the action of vanadium as an inhibitor of cholesterol synthesis alone. While there is no doubt that the major effect of vanadium on lipids is its inhibition of cholesterol synthesis, inhibition of lipogenesis *in vitro* (3) and of phospholipid synthesis *in vitro* and *in vivo* have been reported (25).

Thus, from the data in this paper it can be reasonably concluded that through inhibition of endogenous cholesterol synthesis by a specific inhibitor of that process it is possible to produce a lowering of tissue cholesterol stores. With specific inhibitors of cholesterol synthesis a practical means is available to test the widely held hypothesis that reduction of body cholesterol content may modify the course of atherosclerosis and, in particular, coronary atherosclerosis. Such tests would require long term studies on large numbers of individuals. The effect of reduction of body cholesterol stores could then be determined from the incidence of myocardial infarction and survival of the treated group. In this manner a definitive answer to the correctness of the original hypothesis can finally be obtained.

SUMMARY

1. The effect of a cholesterol synthesis inhibitor (vanadium) on sterol balance in five young men on a fixed diet has been studied.

2. Inhibition of endogenous cholesterol synthesis in man for six weeks resulted in a statistically significant lowering of the serum total and free cholesterol levels and of the C/P ratio. Concomitantly there was a significant reduction in fecal total sterol excretion.

3. A method is presented for the calculation of the dynamics of tissue sterol storage from sterol balance data. By this means it is shown that inhibition of endogenous cholesterol synthesis produced a lowering of tissue cholesterol stores in four of the five men studied.

ACKNOWLEDGMENTS

The authors wish to express their graditude to Dr. R. P. Cook of St. Andrews University, Dundee, Scotland and Dr. H. J. Grady of the University of Kansas for their aid in reviewing the calculations. Without the helpful advice and competent supervision of Mrs. Jack McCabria, the necessary rigidity of the diets could not have been maintained. The authors are likewise indebted to Misses Patricia Unangst and Mary Ruth Dietrich for valuable technical assistance.

ADDENDUM

Sample calculation for B. B., sixth week of vanadium period, from data in Table I

1. ^{A}D = neutral sterol fecal content of control period minus neutral sterol fecal content of experimental period (21).

$^{A}D_R = (676 + 152) - (606 + 56) = 166$ mg. per day.

$^{A}D_V = (676 + 152) - (510 + 40) = 278$ mg. per day.

$^{AF}D = 0.25 \times {}^{A}D$ (22).

$^{AF}D_R = 0.25 \times 166 = 42$ mg. per day.

$^{AF}D_V = 0.25 \times 278 = 70$ mg. per day.

$740 - {}^{A}D + I$ = gut sterol output.

$I_R = (606 + 56 + 499 + 166 - 740) = 587$ mg. per day.

$I_V = (510 + 40 + 472 + 278 - 740) = 560$ mg. per day.

2. $I_R = S_R + {}^{AF}D_R + \Delta B_R$, where $\Delta B_R = 0$; $S_R = 587 - 42 = 545$ mg. per day.

3. $\frac{S_V}{S_R} = \frac{I_V}{I_R}$; $S_V = 545 \times \frac{560}{587} = 520$ mg. per day.

4. $I_V = S_V + {}^{AF}D_V + \Delta B_V$; $\Delta B_V = 560 - 520 - 70 = -30$ mg. per day.

5. To calculate ΔSe_V:

$$Se_R = 1{,}410 \times \frac{146}{2.2} \times 0.05 = 4{,}681 \text{ mg. in total serum at end of recovery period;}$$

$$Se_V = 1{,}380 \times \frac{147}{2.2} \times 0.05 = 4{,}610 \text{ mg. in total serum at end of vanadium period; and}$$

$$\Delta Se_V = \frac{4{,}610 - 4{,}681}{21} = -3 \text{ mg. per day.}$$

6. $\Delta B = \Delta T + \Delta Se$; $\Delta T_V = -30 - (-3) = -27$ mg. per day.

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ESTIMATION OF PULMONARY RESISTANCE BY REPETITIVE INTERRUPTION OF AIRFLOW

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It is often desirable in the study of pulmonary function to know the frictional or resistive forces associated with movement of the lungs and flow of gas in the airways. The magnitude and sign of these forces, expressed as resistive pressure differences, change with the rate and direction of volume displacement, expressed as flow. The ratio of resistive pressure difference to flow is termed pulmonary resistance and includes the "viscous resistance" of the lung tissue and the "viscous and turbulent resistance" of the airways. It does not include elastic and inertial effects of the lungs and their contained air, nor does it include mechanical effects of the chest wall, diaphragm and functionally related structures involved in respiratory motion. Defined in this way pulmonary resistance relates to the quality and quantity of moving lung and to patency of the airway as a whole.

Several methods exist for the estimation of pulmonary resistance. The most direct involves the measurement of intrapleural pressure, airflow and tidal volume. The resistive pressure gradient is that component of intrapleural pressure remaining after subtraction of the pressure necessary to maintain the lung statically at a given volume. When related to airflow it determines pulmonary resistance. A less direct but safer and more practicable technique substitutes esophageal pressure for intrapleural pressure (1). Still less direct but more convenient is the interrupter method in which the rapidly equilibrated pressure at the mouth following sudden obstruction of flow is taken as a measure of the nonelastic component of transpulmonary pressure. The validity of this method depends upon the assumptions that pressure recorded at the mouth rises to equal alveolar pressure (as altered) when motion of air ceases, and that intrapleural pressure does not appreciably change as a result of airflow interruption. Thus, the interrupter method is expected to measure the sum of airway and lung tissue resistance. Several investigators have designed apparatus for this type of resistance measurement and have interpreted their results variously (2, 3, 4). Mead and Whittenberger examined the technique critically, setting forth the theoretical basis and comparing it with the esophageal balloon method (5). In their group of healthy subjects, the method of single interruption slightly overestimated pulmonary resistance.

The present investigation evaluates repetitive interruption of flow as a means of determining pulmonary resistance using a simple portable apparatus. This communication deals with details of the method and its comparison with two other methods of measuring pulmonary resistance.

METHOD AND THEORY OF METHOD

The apparatus.[1] The repetitive interrupter consists of two concentric metal tubes having radial slots 90° apart (Figure 1). As the inner tube rotates, airflow is switched on and off ten times per second for equal intervals of time. A third concentric tube restricts the egress of gas and forms the nonlinear flowmeter. The transition time between flow and interruption is short compared to the half-cycle time. As the subject breathes through the interrupter, pressure and flow rapidly equilibrate in the open and closed intervals so that the oscillographic tracing of mouth pressure exhibits "square waves" (Figure 2). The lower plateau (P_1) in the tracing measures the pres-

* This work was done during tenure of an Established Investigatorship of the American Heart Association.

[1] A direct-writing instrument using the interrupter principle and arranged to give a plot of pulmonary resistance against exhaled volume is available from Meadowbrook Instrument Company, 1440 Carol Road, Meadowbrook, Pa.

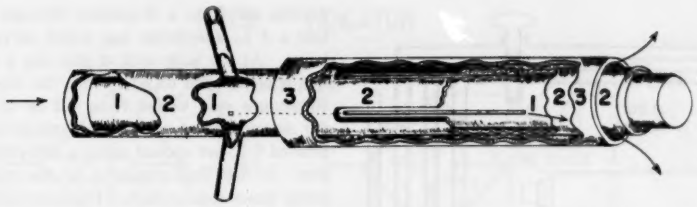

FIG. 1. ROTARY VALVE

Air flows from left to right through stainless steel rotor (1), radially through slots in rotor and brass stator (2) and from left to right through annular space between stator and brass sleeve (3). Rotor is turned at a constant speed of 150 rpm. Scanning holes at left end of rotor connect pressure taps at left end of stator to airway, one at the middle of open phase, the other at the middle of closed phase.

sure drop in the nonlinear flowmeter during flow. The upper plateau gives the alveolar pressure at "equilibrium" (P_2) when flow is stopped; the difference between the plateaus is taken as a measure of the nonelastic pressure drop in the lungs and airways (ΔP). Since pulmonary inertance can be neglected, the pressure change at the mouth is equal to the pulmonary resistive pressure gradient, provided intrapleural pressure does not change as a result of interruption.

Nonlinear flow measurement. In the following paragraphs these symbols will be used: F, flow; ΔP, the resistive pressure gradient; R_p, the coefficient of pulmonary resistance (including viscous and turbulent components); R_v, a coefficient of viscous resistance; R_t, a coefficient of turbulent resistance; P_1, the pressure gradient across the nonlinear flowmeter; and R_e, the coefficient of resistance of the nonlinear flowmeter.

The motion of air and tissues is made up of viscous tissue motion, laminar (viscous) airflow and turbulent airflow. Where flow is viscous, the resistive pressure difference is linearly related to airflow ($\Delta P = R_v \times F$). Where flow is turbulent the resistive pressure gradient is approximately proportional to the square of flow ($\Delta P = R_t \times F^2$). When viscous and turbulent flow exist in combination, the resistive pressure gradient is related to some power of flow between 1 and 2 ($\Delta P = R_p \times F^n$, $1 < n < 2$). Ainsworth and Eveleigh (6) made a noteworthy contribution to the understanding of pulmonary resistance by finding that the formulation, $\Delta P = R_p \times F^{1.6}$, was adequate to describe normal individuals. After showing that the nonlinearity of the pressure-flow relationship was characteristic of the population rather than of the individual they were able to express differences between the individuals and change within an individual following a broncho-motor drug entirely in the magnitude of the resistance coefficient. By constructing a flowmeter, the nonlinear characteristic of which matched that of the respiratory tract, they could estimate pulmonary resistance from the ratio of internal (pleura-to-mouth) and external (nonlinear flowmeter) resistive pressure gradients, thus ingeniously simplifying the interrupter method.

Since the nonlinear flowmeter is constructed according to the relationship:

$$P_1 = R_e F^{1.6},$$

and since the relationship of pressure to flow in the subject's lungs and airway is

$$\Delta P = R_P \times F^{1.6},$$

it follows that

$$\frac{\Delta P}{P_1} = \frac{R_P \times F^{1.6}}{R_e \times F^{1.6}}, \quad or \quad R_P = \frac{R_e \times \Delta P}{P_1}.$$

Thus, measurement of the pressure ratio $\Delta P/P_1$ and calibration of the nonlinear flowmeter (determination of R_e) suffice for calculation of pulmonary resistance.

Recording of pressure. While the pressure ratio could be determined from oscillographic tracings, it was desirable to simplify the technique further. Consequently, the pressure leads were valved in such a way that pressure was applied to the manometers during only the middle third of each half-cycle and the manometer sealed off for the remainder of the cycle. One lead registered mouth

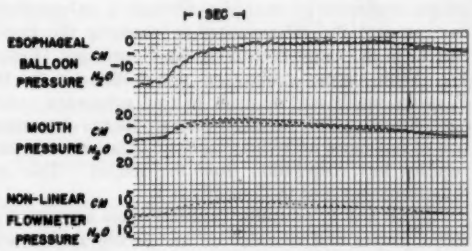

FIG. 2. ESOPHAGEAL PRESSURE, MOUTH PRESSURE AND AIRFLOW DURING REPETITIVE INTERRUPTION IN EXPIRATION

The "square wave" pattern of mouth pressure is shown. Small oscillations occur in esophageal pressure at a frequency of 10 cycles per second.

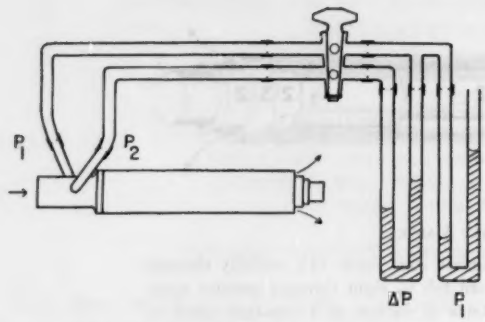

FIG. 3. THIS FIGURE SHOWS THE MANNER OF CONNECTING MANOMETERS THROUGH A DOUBLE STOPCOCK TO THE ROTATING VALVE

pressure during the middle third of the interrupted phase (P_2) and the other lead registered pressure during the middle third of the flow phase (P_1). The valving was accomplished by placing "scanning holes" of precise dimensions and location in the rotating and stationary inner cylinders. Thus, with steady average flow, the pressures in the two valved or "scanned" leads are constant at the plateau values and may be indicated on slowly responding systems such as water manometers. The scanned pressures have been recorded in several ways for indicating pulmonary resistance. Three will be described.

If a double stopcock is interposed in the pressure leads, it can be closed during the subject's exhalation into the interrupter, thus holding the plateau pressures in the manometers for accurate reading (Figure 3). P_1 appears on one manometer; P_2 minus P_1 or ΔP is registered on the other (differential) manometer. The ratio is calculated and used as above. By plotting ΔP against P_1 at different flows one can discover how well pulmonary resistance and flow meter resistance are matched in nonlinearity (Figure 4). This arrangement was used in Comparison 2.

If the scanned pressure is applied to balanced strain gauges connected in opposition through a galvanometer one can zero the galvanometer by changing the supply current in the gauge registering the P_1 pressure (Figure 5). With proper calibration, the milliameter in the P_1 gauge supply may be made to read pulmonary resistance directly. This arrangement permits the determination of resistance with changing flow, is faster in use and requires less cooperation from the subject. This arrangement was used in Comparison 1.

The ΔP and P_1 pressures (scanned) may be applied to strain gauges and recorded on an oscillograph as was done in Comparison 3. This permitted simultaneous volume recording so that comparisons of esophageal balloon and interrupter methods could be made at the same lung volume.

Calibration. The entire instrument was calibrated at a mean flow of 0.5 L. per second (half-cycle flow 1.0 L. per second, as air flowed only half the time) by conducting air from a flowmeter through a high resistance into a 4 L. anesthesia bag which served as a lung analogue. At the wide neck of this bag a variable resistance was attached and distal to this, the repetitive interrupter. Resistance was varied from 0.2 to 15 cm. H_2O per L. per second and at each setting measured first at a steady flow of 1 L. per second using a differential water manometer. It was then measured by the repetitive interrupter using water manometers. Pressure in the anesthesia bag, analogous to alveolar pressure in this calibration scheme, varied less than 0.1 cm. H_2O on interruption.

PROCEDURES

Comparison 1 (*Table I*). Ten patients were prepared for elective thoracotomy with Demerol®, Pentothal Sodium® and succinylcholine chloride. After intubation with a cuffed tracheal tube, they were maintained on a nitrous oxide-oxygen mixture and on succinylcholine by intravenous drip. The tracheal tube was connected by a valve either through the anesthesia machine or through the apparatus for the measurement of resistance. Following dissection down to the parietal pleura, a curved cannula with several perforations was inserted into the intrapleural "space." Ten to twenty cc. of air was admitted to the chest via the cannula and transpulmonary pressure recorded using a differential strain gauge and direct-writing oscillograph. The airway was connected to a screen pneumotachometer and airflow recorded. The flow signal was integrated electrically to generate a tracing of tidal volume. Recordings were made of passive expirations and resistance calculated by the method of Otis, Fenn and Rahn (7, 8). Alternately, passive expirations were directed into the repetitive interrupter for determination of resistance using strain gauges and the ratio circuit shown in Figure 5. The resistance of the

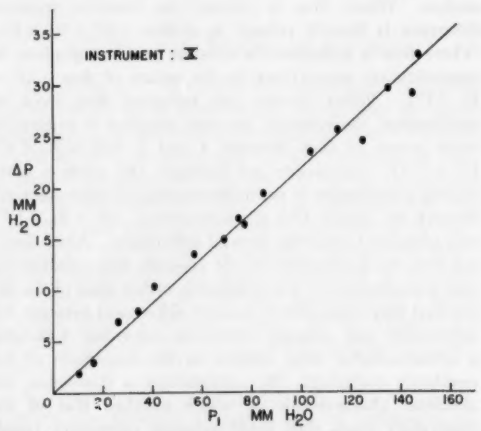

FIG. 4. PLOT OF "PULMONARY RESISTIVE" PRESSURE GRADIENT (ΔP) AGAINST FLOWMETER PRESSURE GRADIENT (P_1) OVER THE FLOW RANGE 0.1 TO 1.9 L. PER SECOND

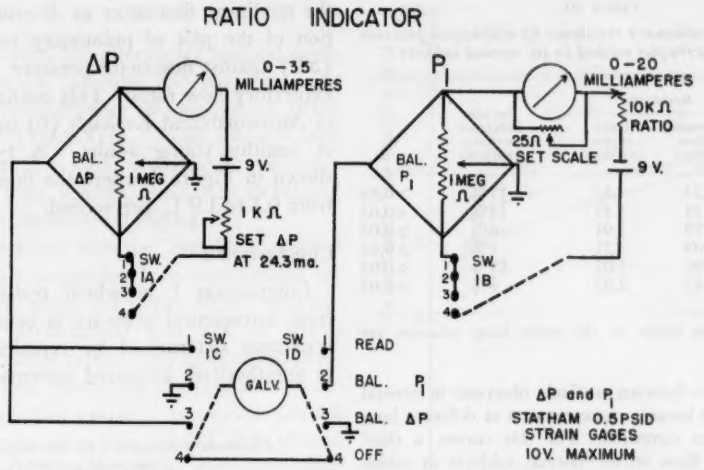

FIG. 5. CIRCUIT FOR DETERMINING THE RATIO OF TWO PRESSURES, USING 0.5 PSID STRAIN GAUGES

Proper adjustment allows pulmonary resistance to be read directly from the 20 ma. meter.

endotracheal tube was measured and subtracted from both estimates of pulmonary resistance.

Comparison 2 (Table II). In a second study resistance values by repetitive interruption and by the esophageal pressure method were compared in 11 healthy young adults. The subjects were studied in the sitting position and transpulmonary (esophagus-to-mouth) pressure, tidal volume and airflow were recorded continuously during spontaneous breathing. The nonelastic component of transpulmonary pressure at the instant when flow was equal to one L. per second was taken as the value of pulmonary resistance. On a subsequent day, resistance was detemined by repetitive interruption using water manometers to determine values of ΔP and P_1 (Figure 3). Ten breaths were analyzed by each method yielding 20 resistance values by the esophageal pressure method and 10 values by the interrupter method. Averages and standard errors are shown in Table II.

Comparison 3 (Table III). The lung volumes at which interrupter measurements were made in Comparison 2 were not determined. Because resistance varies considerably with lung volume (1, 9, 10), it seemed likely

TABLE I

Comparison 1: Pulmonary resistance determined by repetitive interruption compared with resistance calculated from intrapleural pressure, volume and flow recordings during passive expiration in ten anesthetized patients with pulmonary tuberculosis

Patient	Resistance by interrupter method	Resistance by intrapleural pressure method
	cm. H_2O/L./sec.	*cm. H_2O/L./sec.*
M. L.	4.6	4.8
F. C.	7.7	8.3
E. N.	3.9	3.7
A. W.	6.8	7.1
H. C.	2.3	3.3
J. C.	2.8	2.5
W. J.	2.9	2.5
E. M.	3.2	2.9
A. R.	5.5	5.0
W. R.	5.0	4.6

TABLE II

Comparison 2: Pulmonary resistance in eleven healthy adults as determined by measurement of esophageal pressure and by repetitive interruption * †

Subject	Esophageal balloon method ± S. E.	Interrupter method ± S. E.	p
J. C.	2.17 ± 0.07	2.00 ± 0.07	>0.05
J. S.	1.92 ± 0.07	1.13 ± 0.09	<0.05
G. G.	1.84 ± 0.10	2.24 ± 0.24	>0.05
R. W.	2.01 ± 0.12	1.61 ± 0.06	<0.05
E. B.	1.80 ± 0.11	1.79 ± 0.06	>0.05
B. B.	1.99 ± 0.12	2.21 ± 0.32	>0.05
W. O.	1.22 ± 0.11	1.84 ± 0.03	<0.05
J. A. C.	1.48 ± 0.12	1.19 ± 0.06	<0.05
W. J. W.	2.18 ± 0.05	2.97 ± 0.04	<0.05
A. B.	0.89 ± 0.14	2.14 ± 0.9	<0.05
M. B. M.	3.54 ± 0.15	2.21 ± 0.09	<0.05
Group averages	1.91	1.94	

* Means and standard errors were calculated from twenty resistance values by the esophageal pressure method and ten values by the interrupter method.

† Resistance in cm. H_2O per L. per second.

TABLE III

Comparison 3: Pulmonary resistance by esophageal pressure method and interrupter method in six normal subjects *

Subject	Resistance: Esophageal balloon method	Resistance: Interrupter method	Per cent difference between methods	p
G. G.	1.53	1.84	17%	>0.05
J. S.	1.21	1.41	14%	<0.05
D. R.	1.79	1.91	6%	>0.05
D. G.	3.09	3.21	4%	>0.05
A. B.	0.91	1.07	15%	<0.05
M. M.	2.81	2.92	4%	>0.05

* Measurements made at the same lung volumes are compared.

that the variation between methods observed in several subjects occurred because measurements at different lung volumes had been compared. For this reason, a third comparison was done on six normal subjects in which volumes were recorded and resistances by the two methods compared at the same lung volume. The results of this comparison are shown in Table III.

RESULTS

The reproducibility of interrupter values of resistance is shown in Tables II, IV and V. Table II shows the resistance values for each subject in Comparison 2 and the standard error of 10 observations in each subject. Table IV presents typical data from one normal subject. Table V shows the resistance change effected by bronchodilator in a subject with mild asthma.

The relationship between resistive pressure and flow in the 11 subjects matched satisfactorily the relationship of resistive pressure to flow across the nonlinear flowmeter as determined by inspection of the plot of pulmonary resistive pressure (ΔP) against flowmeter pressure (P_1) at various expiratory flow rates. This confirms the findings of Ainsworth and Eveleigh (6) in a larger group of healthy young adults. A typical result is shown in Figure 4 where the flow range extends from 0.1 to 1.9 L. per sceond.

TABLE IV

Ten determinations of pulmonary resistance by repetitive interruption in one healthy adult subject (J. C.) showing typical variation

ΔP	P_1	Resistance by interrupter
mm. H_2O	*mm. H_2O*	*cm. H_2O/L./sec.*
27.6	106.1	1.60
32.8	97.2	2.18
30.4	94.6	2.06
25.5	83.0	1.95
26.8	83.9	2.06
32.0	101.3	2.03
32.8	80.2	2.72
19.2	65.1	1.87
29.7	100.8	1.87
33.7	122.0	1.72
	Mean	2.00
	S. D. 0.21	S. E. 0.07

Comparison 1

Comparison 1 in which resistance calculated from intrapleural pressure is compared with the resistance determined by repetitive interruption in anesthetized, intubated patients is summarized in Table I. Even though the resistance of the endotracheal tube and valve was subtracted from all values, the remaining (pulmonary) resistance tended to be above the normal range, perhaps because of bronchospastic reaction to the presence of a cuffed tube in the trachea or to the presence of pulmonary disease, or both. The correlation even at the higher values of resistance is good.

TABLE V

Pulmonary resistance in one subject suffering from mild asthma *

ΔP	P_1	$\Delta P/P_1$	R_1	Average
Before Isuprel®				
44.4	98.4	0.452	3.04	3.50
45.8	104.0	0.431	2.91	
48.4	83.4	0.582	4.07	
57.3	102.5	0.534	3.69	
68.2	123.8	0.551	3.82	
5 minutes after Isuprel®				
33.0	117.0	0.282	1.72	1.82
26.5	85.5	0.310	1.96	
23.6	74.4	0.318	2.03	
28.7	88.7	0.324	2.07	
21.7	95.0	0.228	1.32	

* Measurements taken before and five minutes after inhalation of an aerosol of Isuprel®.

Comparison 2

The first column of Table II summarizes measurements by the esophageal ballon method in 11 normal subjects; the second column summarizes measurements on the same subjects by the interrupter technique. Though there was reasonable agreement between the two methods, significant differences were encountered in seven of the 11 subjects.

Comparison 3

In all six subjects the interrupter method overestimated resistance (average 10.5 per cent). In two of the six the difference was significant at the 5 per cent level (Table III). In all subjects both methods showed the same changes in resistance with changing lung volume, resistance increasing as lung volume decreased (Figure 6).

The best correlation with the "standard" method was obtained in Comparison 1 on anesthetized relaxed patients in whom the upper airway was bypassed by an intratracheal tube and in whom intrapleural pressure rather than esophageal pressure was recorded. Variation of upper airway resistance beyond the subject's conscious control or discrepancy between esophageal and pleural pressures or both of these factors may contribute to the greater variation between methods observed in Comparisons 2 and 3.

Comparison 3 in which resistances were compared at similar lung volumes gave a better correlation between methods than Comparison 2 where the lung volume at which the interrupter values were obtained was not accurately known. This suggests that the variation of resistance as a function of lung volume accounts for part of the variation between methods observed in Comparison 2.

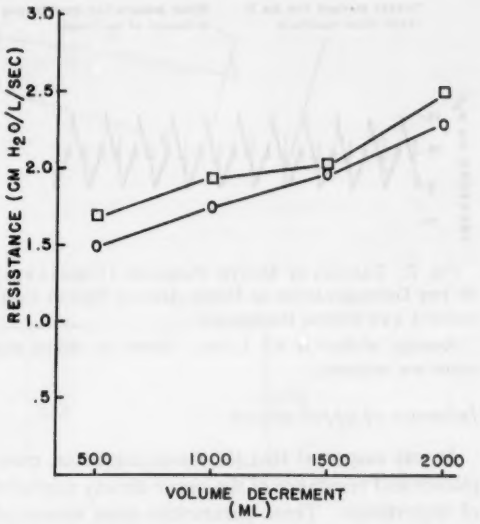

FIG. 6. PULMONARY RESISTANCE BY REPETITIVE INTERRUPTION (SQUARES) AND BY CALCULATION FROM ESOPHAGEAL PRESSURE (CIRCLES)

Abscissa, volume exhaled after maximal inspiration; ordinate, resistance.

DISCUSSION

Analysis of this method of measuring pulmonary resistance and of its sources of error requires that one consider the several components of pulmonary resistance and determine what pressure and flow measurements made at the mouth mean in terms of forces and motion within the whole respiratory system. In addition one should attempt to define the range over which there can be reasonable confidence in the values. In the following sections these considerations will be amplified.

Influence of thorax

As shown by Mead and Whittenberger (5) pressure changes on single interruption are small in the esophagus and presumably in the pleural space. They have presented evidence that this is due to relatively high thoracic compliance which absorbs momentum from the diaphragm and keeps intrapleural pressure nearly constant during interruption. Similarly, only small oscillations occur in esophageal pressure during repetitive interruption (Figure 2). Thus the thorax, diaphragm and abdominal contents act together at 10 cycles per second as a low impedance pressure source and only a small component of "extrapulmonary resistance" is added to that of the lungs and airways under the usual conditions of measurement. This added component amounts to 0.1 to 0.2 cm. H_2O per L. per second and increases the change of alveolar pressure with interruption. It results in an overestimation of pulmonary resistance by 5 to 10 per cent. At extremes of lung volume, changes of esophageal pressure on interruption are greater than in the mid-range of volume, indicating that the thorax no longer compensates for inertance of the diaphragm and abdominal contents so effectively. This stiffening of the chest (lowered thoracic compliance) is probably due to the tension of intercostal muscles and the approach of the thorax to its volume limit, and may result in more significant overestimation of pulmonary resistance by interruption.

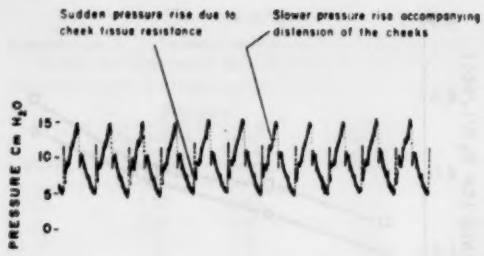

FIG. 7. TRACING OF MOUTH PRESSURE (UNSCANNED) IN THE DETERMINATION OF UPPER AIRWAY SHUNT COMPLIANCE AND TISSUE RESISTANCE

Average airflow is 0.5 L./sec. Glottis is closed and nares are occluded.

Influence of upper airway

It was suspected that the tissue inertance, compliance and resistance of the upper airway might be of importance. These parameters were measured in two normal subjects. This was done by conducting air into one nostril at 0.5 or at 1 L. per second while the subject, with the other nostril and the glottis closed and with lips tightly around the mouthpiece of the interrupter, allowed air to pass from the mouth through the interrupter. Mouth pressure was recorded on an oscillograph (Figure 7) and from the tracings obtained tissue compliance and resistance of the upper airway were calculated. Compliance was 0.001 L. per cm. H_2O and tissue resistance was 5 cm. H_2O per L. per second, with cheeks relaxed. Inertance was calculated from the frequency of oscillations occurring at valve closure and found to be negligible.

Simplified electrical and pneumatic analogues were set up for clarification of the basic physical problem, using the values given in Table VI, according to the scheme of Figure 8. These analogues were arranged so that "pulmonary resistance" could be varied from 0.5 to 20 cm. H_2O per L. per second and estimates with steady flow compared to those with pulsating flow. It was evident from the behavior of the analogue that "shunt" compliance and resistance in the upper airway influence the pattern of mouth pressure significantly. The distensible upper airway must receive gas as pressure in the mouth rises to equal alveolar pressure during equilibration. The more distensible the upper airway (mainly the cheeks) and the greater the *pulmonary* resistance, the longer this equilibration requires. Fortunately, the upper airway is relatively rigid (low compliance, high tissue resistance and thus high impedance), and should permit estimation of normal pulmonary resistance within 2 per cent, judging from the behavior of the pneumatic analogue.

Figure 8 shows the extent to which the method underestimates resistance as pulmonary resistance increases. Pulsatile or interrupter resistance was calculated and compared to steady flow resistance at several levels of increased resistance. These calculations were based upon the differential equations describing pulsatile flow at 10 cps in electrical and pneumatic analogues schematized in Figure 8. The nonlinear relationship of the pulmonary resistance pressure to flow was found to shorten the effective equilibration time and calculations taking this into account agreed with values observed in a pneumatic analogue having similar nonlinear characteristics. Further calculations assuming a linear flow-pressure relationship showed that resistance was underestimated to a greater extent than in the case of nonlinear resistance.

TABLE VI

Components with their values and units used in electrical and mechanical analogues of the respiratory system *

Component	Symbol	Mechanical (pneumatic) analogue	Electrical analogue
Lung compliance	C_l	0.200 L./cm. H_2O	200 microfarads
Lung gas compliance	Cg	0.004 L./cm. H_2O	4 microfarads
Upper airway shunt compliance	Cm	0.001 L./cm. H_2O	1 microfarad
Airway resistance	R_a	1.5 to 20 cm. H_2O L./sec.	1,500 to 20,000 ohms
Lung tissue resistance	R_l	0.5 cm. H_2O L./sec.	500 ohms
Pulmonary resistance ($R_a + R_l$)	R_p	2.0 cm. H_2O L./sec.	2,000 ohms
Upper airway shunt resistance	R_m	5.0 cm. H_2O L./sec.	5,000 ohms
Flowmeter resistance	R_s	6.4 cm. H_2O L./sec.	6,400 ohms
Pulmonary gas inertance	I_a	0.01 cm. H_2O L./sec./sec.	10 henries

* The components of pneumatic and electrical analogues based on known physical properties of the human respiratory system. Both analogues follow the schematic diagram shown in Figure 8.

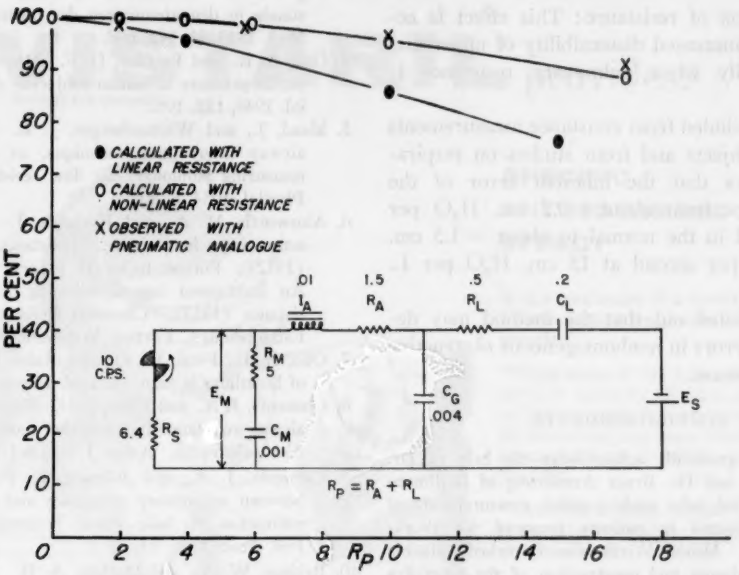

FIG. 8. PLOT OF "PULMONARY RESISTANCE" MEASURED AT 1 L. PER SECOND STEADY FLOW AND 0.5 L. PER SECOND PULSATILE FLOW IN A PNEUMATIC ANALOGUE CONSTRUCTED ACCORDING TO THE DIAGRAM SHOWN IN THE INSERT

Parameters are based on actual measurements of mechanical properties of the human respiratory system. Ordinate, percentage of steady flow resistance measured by interrupter technique; abscissa, steady flow resistance at 1 L. per second. Symbols with units and electrical equivalents are defined in Table VI.

Thus the nonlinearity of pulmonary resistance increases the apparent accuracy of the interrupter method. The degree of nonlinearity used in calculations and the analogue was comparable to that measured in healthy adults. Figure 8 shows that pulmonary resistance up to 15 cm. H_2O per L. per second in the analogue is measured with a deficit of less than 10 per cent.

To what extent linearity is altered in patients with elevated resistance and the effect of such change on the validity of the interrupter method remains to be elucidated. As Mead, Lindgren and Gaensler (11) and Fry, Ebert, Stead and Brown (12) have shown, pressure equilibration following interruption of airflow is comparatively slow in patients with obstructive emphysema. Cheng, Godfrey and Shepard have used repetitive interruption and recording of exhaled volume in studies of patients with obstructive pulmonary disease (13). They have found resistance-volume patterns that suggest the mechanisms of obstruction and are hence of diagnostic value. The failure of complete pressure equilibration following interruption of flow in these patients did not seriously detract from the clinical usefulness of the method.

SUMMARY

1. A simple technique for estimation of lung and airway resistance has been developed using repetitive interruption of airflow and nonlinear flowmetering.

2. The method is satisfactory with respect to reproducibility of values and is a valid method for measuring resistance as judged by comparison with standard methods.

3. The theory of the method has been briefly explored with special attention to the influence of the thorax and effect of upper airway properties. The physical properties of the thorax cause slight overestimation of resistance and this effect is increased at the extremes of lung volume. The effect of upper airway properties is to cause

underestimation of resistance: This effect is accentuated by increased distensibility of upper airways especially when pulmonary resistance is high.

4. It is concluded from resistance measurements on human subjects and from studies on respiratory analogues that the inherent error of the method ranges from about + 0.2 cm. H_2O per L. per second in the normal to about − 1.5 cm. H_2O per L. per second at 15 cm. H_2O per L. per second.

5. It is pointed out that the method may develop larger errors in nonhomogeneous obstructive pulmonary disease.

ACKNOWLEDGMENTS

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